Radioimmunotherapy

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Radioimmunotherapy
in head and neck cancer patients

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Radioimmunotherapy

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door

David Raoul Colnot

geboren te Bergen (N.H.)

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To my parents
To Margit and Amélie

Contents

1

GENERAL INTRODUCTION

1. HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC)

1.1 Incidence of HNSCC

Squamous cell carcinoma is by far the most frequently occurring malignant tumor in the head and neck. More than 95 % of HNSCC originate from the mucosal linings of the oral cavity, the pharynx, or the larynx. Risk factors for developing HNSCC are smoking and alcohol consumption, oncogenic human papillomavirus (HPV), but also tobacco or betel quid chewing. The estimated worldwide incidence of HNSCC in 2000 was more than 551,000 cases, and more than 217,000 deaths (*1*). HNSCC is responsible for 12 and 7 % of male and female cancers, respectively, in the developing world. For the developed countries these figures are 7 and 2 %, respectively (*2*). In the USA, more than 37,000 new HNSCC cases and 12,000 deaths are expected for 2003 (*3*). A rise in incidence rates of HNSCC among males is noticeable in Europe and the USA (*2*). For oral tongue cancer an increase in incidence for young adults is observed over the last decades (*4-6*). The total incidence of HNSCC in the Netherlands in 1998 was 2300 cases, according to the National Cancer Registry (*7*). HNSCC was on the sixth position among the most frequent invasive tumors for males, and for the male age group of 45-59 years it was the fourth most common cancer. In total, 735 patients died of HNSCC in the Netherlands (*7*). Over the last decade, the incidence of primary tumors of the pharynx and esophagus in the Netherlands has increased for both sexes, due to changes in tobacco and alcohol habits. In contrast, a 20 % reduction over the last decade was observed for the incidence of laryngeal cancer for males, due to the decrease in smoking habits in preceding decades (*8*).

1.2 Staging, treatment, and prognosis of patients with HNSCC

Classification and staging of HNSCC is performed according to the TNM system of the International Union Against Cancer (Union Internationale Contre le Cancer, UICC) (*9*). The TNM classification system describes the anatomical extent of the primary tumor, the status of the regional lymph nodes, and the presence or absence of distant metastases. Based on the TNM classification, overall clinical stages I to IV can be assessed. Stage groups I and II refer to small primary tumors without metastatic spread to regional lymph nodes in the neck or metastases at distant sites, whereas stages III and IV refer to patients with large primary tumors or the presence of lymph node metastases or metastases at distant sites. About one third of the

patients present with stage I and II disease, while two third of the patients present with disease stages III and IV (*10*).

Initial therapy of HNSCC consists of surgery and/or radiotherapy. While early stages I and II are generally treated with surgery or radiotherapy alone and have a good prognosis, the more advanced stages III and IV are treated with combined surgery and radiotherapy and have a high failure rate, either locoregionally or at distant sites. Despite improvements in locoregional control for patients with advanced HNSCC, the overall survival rates of HNSCC patients have not improved significantly over the last 25 years. For oral cavity cancer, the relative 5 year survival rate in the USA remained about 55 %, and for laryngeal cancer this number was 66 % (*3*).

Table 1. Trends in five-year relative survival rates (%) by year of diagnosis, USA, 1974-1998.

Site	Relative 5-year survival rate (%)		
	1974-76	**1983-85**	**1992-98**
Larynx	66	67	64
Oral Cavity	53	53	56

Source: Surveillance, Epidemiology, and End Results Program, 1973-1999, Division of Cancer Control and Population Sciences, National Cancer Institute, Bethesda, MD, 2002 (*3*).

Although few HNSCC patients present with distant metastases at initial diagnosis, the development of distant metastases after locoregional treatment in large series of patients ranges between 5-25 % (*11-15*). In subgroups of patients with advanced disease, the incidence of distant metastases can be as high as 50 % (*12-16*). The most important prognostic indicator for distant metastases of HNSCC is the extent of metastatic spread to lymph nodes in the neck. Factors like the localization,

size and number of lymph node metastases, but also the presence of extranodal spread influence the prognosis of HNSCC patients (*16-23*).

The addition of chemotherapy, either given in adjuvant or neoadjuvant setting, to the primary treatment of HNSCC has resulted in improved quality of life and considerable advances in terms of organ preservation, but not in benefit in survival of these patients (*24-29*). An effective systemic adjuvant treatment is needed in order to improve survival rates in HNSCC patients.

2. Monoclonal Antibodies

2.1 History

Among novel therapeutic approaches is the use of monoclonal antibodies (MAbs). The concept of developing antibodies selectively reactive with tumor cells dates from the early 1900s, when Ehrlich envisioned "magic bullets", able to recognize specific structures on tumor cells and, when loaded with toxic agents, able to kill these tumor cells. The introduction of the hybridoma technology by Kohler and Milstein in 1975 allowed the development of uniform MAbs specifically directed against virtually any particular cellular antigen (*30*). In this way, MAbs could be produced for a variety of applications: selective inhibition of proliferation of tumor cells by blocking growth factors or growth factor receptors, induction of apoptosis, recruitment of immunological effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and the selective delivery of cytotoxic payloads to tumor cells such as radionuclides and toxins. In recent years, several MAbs have been approved by the U.S. Food and Drug Administration (FDA) for therapeutic application, among which are two radiolabeled MAbs, ^{90}Y-ibritumomab tiuxetan and ^{131}I-tositumomab. The first MAb for treatment of cancer was approved by the FDA in 1997, and since then another seven have been approved predominantly for treatment of hematological malignancies. As depicted in Table 2, several other MAbs designed for treatment of cancer are in advanced stages of clinical investigation, that is, in phase III trials, among which there are two radiolabeled MAbs. Moreover, several MAbs have been approved by the FDA for other therapeutic applications, such as treatment of rheumatoid arthritis, cardiovasvular diseases, transplantation rejection, asthma, and infectious disease.

2.2 Characteristics of monoclonal antibodies

Several MAbs have been developed with different characteristics and clinical applications. The use of enzyme digestion and recombinant-DNA technology has made it possible to create different types of antibodies, antibody fragments and constructs, with molecular weights ranging from 20 to 150 kDa. To achieve optimal exposition of tumor tissue, MAbs should demonstrate a high and selective uptake in tumor tissue, and a long tumor residence time. In general, the uptake of MAbs in tumor tissue after intravenous administration ranges between 0.001 and 0.01 % of the injected dose per gram of tissue (%ID/g). Numerous factors influence tumor uptake of MAbs. Among these are type, size, antigen binding (affinity), and pharmacokinetics of the MAb, tumor vascularity and intratumoral pressure, and the level of expression of antigens in tumor tissue.

Table 2. Monoclonal antibodies for cancer therapy.

A. FDA approved MAbs						
Product	**Company**	**Type of antibody**	**Target antigen**	**Mechanism**	**Disease**	**Date of approval**
Rituximab (Rituxan®)	IDEC, Genentech, Roche	chimeric IgG1	CD20	ADCC, CDC, apoptosis	NHL	26/11/97
Trastuzumab (Herceptin®)	Genentech	humanized IgG1	HER2	Inhibition of HER2 mediated poliferation	Breast cancer (combined with chemotherapy)	25/9/98
Gemtuzumab Ozogamicin (Mylotarg®)	Wyeth-Ayerst Celltech	humanized IgG1	CD33	Delivery of cytotoxic calicheamine	AML	17/5/00
Alemtuzumab (Campath®)	Millenium	humanized IgG1	CD52	ADCC, CDC	CLL	7/5/01
Ibritumomab Tiuxetan (Zevalin®)	IDEC Schering	murine IgG1	CD20	^{90}Y radiation, ADCC, CDC, apoptosis	NHL	19/2/02
Tositumomab (Bexxar®)	Corixa GlaxoSmithKline	murine IgG2a	CD20	^{131}I radiation, ADCC, CDC, apoptosis	NHL	30/6/03
Cetuximab (Erbitux®)	Imclone, Bristol Myers Squibb, Merck	chimeric IgG1	EGFR (HER1)	Inhibition of cell invasion, proliferation, metastasizing, angiogenesis	Colorectal cancer	12/2/04
Bevacizumab (Avastin®)	Genentech	humanized IgG1	VEGF	Inhibition of VEGF-induced angiogenesis	Colorectal cancer	15/2/04
B. MAbs in Phase III trials						
TNT-1/B (Cotara®)	Peregrine Pharmaceuticals	chimeric IgG1	DNA antigens	^{131}I radiation, targets tumor necrosis	Glioma	Phase III
Mitomomab (BEC-2)	Imclone, Merck	murine IgG2a	GD3	Anti-idiotypic vaccine MAb directed against GD3	SCLC	Phase III
Pemtumomab (Theragyn®)	Antisoma, Roche	murine IgG1	MUC-1	^{90}Y radiation	Ovarian cancer	Phase III
CeaVac®	Titan Pharmaceuticals	murine IgG	CEA	Anti-idiotypic vaccine MAb directed against CEA	Colorectal cancer	Phase III
Ovarex®	Altarex	murine IgG	CA 125	Immune response against CA 125	Ovarian cancer	Phase III
Epratuzumab (LymphoCide®)	Immunomedics, Amgen	humanized IgG1	CD22	ADCC	NHL	Phase III

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cyotoxicity; NHL, non-Hodgkin's lymphoma; AML, acute myelocytic leukemia; CLL, chronic lymphocytic leukemia; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

The most commonly used MAbs for cancer therapy, radiolabeled MAbs included, are intact immunoglobulins (IgG), with a molecular weight of approximately 150 kDa. In humans, intact MAb molecules have a long residence time ranging from a few days to over three weeks, with in general longer residence times for human than for murine MAbs. This results in high MAb concentrations at tumor sites, with optimal tumor-to-nontumor ratios after 2-4 days. A disadvantage is that the slow blood clearance also results in significant exposure to normal organs, especially to the bone marrow. In addition, the large size of a MAb molecule might cause heterogeneous uptake in the tumor because of slow diffusion from the vasculature into and throughout the tumor.

For many years, the use of MAb fragments has been considered a promising alternative. Fragments can be produced out of intact MAb molecules by enzymatic digestion. The resulting fragments are called $F(ab')_2$ (~100 kDa), F(ab') (~50 kDa), or Fab (~40 kDa). By recombinant-DNA techniques even smaller fragments can be made, like single chain Fv (scFv, ~25 kDa). The use of MAb fragments in patients makes blood clearance faster. This results in higher tumor-to-nontumor ratios at earlier time points, and better and more homogeneous penetration into tumor masses. Moreover, fragments are lacking the Fc portion and, therefore, in general are less immunogenic. An important disadvantage of fragments in comparison to intact MAbs, however, is their lower absolute tumor uptake. The aforementioned characteristics in general make intact MAbs first choice for use in therapeutic applications and fragments first choice for use in diagnostic approaches.

Selection of high-affinity MAbs might seem desirable for optimal tumor targeting, but this does not always result in improved tumor uptake. High-affinity can impede the penetration of MAbs deeper into the tumor by their concentrated accumulation at the periphery of the tumor (*31,32*). An intermediate-affinity, therefore, MAb might be more suitable for optimal tumor targeting (*33*). In patients with soluble tumor antigen in blood, high affinity of the MAbs also can affect the pharmacokinetics and tumor targeting of the MAb (*34,35*).

2.3 Immunogenicity of monoclonal antibodies

Most MAbs available nowadays, have been generated by hybridoma technology and originally were murine. One of the major limitations of using murine MAbs (mMAbs) in patients is immunogenicity. Development of human-anti-mouse antibodies (HAMA) can lead to allergic reactions like anaphylaxis, and may lead to rapid clearance of the antibody (*36,37*). Repeated administrations of mMAbs for multiple treatment strategies, therefore, can be limited by the development of HAMA. Advances in DNA technology have allowed engineering of chimeric and humanized

MAbs. Chimeric MAbs (cMAbs) possess murine variable domains and human constant domains (*38,39*). If the immune reaction is directed against the variable domain of the mMAb, however, the construction of a cMAb will not avoid immunogenicity. Development of human-anti-chimeric antibodies (HACA) was indeed noted in a considerable number of patients after administration of cMAbs (*40-44*). This problem can be circumvented by engineering of humanized MAbs (hMAbs), where the antigen recognition part of the murine MAb, the complementarity-determining regions (CDR), are grafted onto a human immunoglobulin framework. Although formation of human-anti-human antibodies (HAHA) has been observed in studies with humanized MAbs, a decrease or even absence of immunogenicity in comparison with their murine counterparts is mostly noted (*45-47*). Fully human MAbs can be produced by the use of phage display libraries (*48-50*), or by the use of transgenic mice that contain human immunoglobulin gene repertoires (*51-53*).

3. Radioimmunotherapy

Radioimmunotherapy (RIT) is targeted radiotherapy using tumor-selective MAbs labeled with radionuclides. In this way, systemically administered radiation can be selectively delivered to tumor sites irrespective their location in the body while sparing normal tissues. An important advantage of RIT as compared to for example chemotherapy and other MAb-based therapeutic approaches is that radionuclides are able to kill adjacent tumor cells. As a consequence, not every tumor cell has to be targeted by MAbs but can be destroyed by a “cross-fire” effect. Another advantage of RIT is that for effectiveness no internalization of the MAb is required. Moreover, RIT is not affected by multidrug resistance, and not entirely dependent on the competence of the immune system. Finally, RIT can be tailored by imaging and dosimetric evaluations. The success of RIT depends on several factors, including the type of MAb used, the radionuclide coupled to the MAb, and the radiosensitivity of the tumor and normal tissues of the patient.

3.1 Radionuclides for radioimmunotherapy

Selection of the ideal radionuclide for RIT is based on size and features of the tumor and antibody, and the physical characteristics of the radionuclide (*54,55*). Important characteristics of the radionuclide in this regard are its decay properties, such as physical half-life and the types of radiation (α-, β-, and/or γ-), and the abundance of the emitted radiation.

For clinical RIT with intact MAbs, β-emitting radionuclides have been most extensively studied. The path-length or range of β-particles is several millimeters, ~10 to 100 cell diameters, which means that also poorly accessible or antigen-negative tumor cells can be efficiently eradicated. Accompanying γ-emission can be of value, as its opens the possibility for imaging with a gamma camera during the RIT procedure. The physical half-life of a radionuclide, by preference, should be matching with the biological half-life of the MAb used. Besides that, also its chemical properties play a role, e.g. the mode of labeling needed and its fate after conjugate catabolism *in vivo*. Some radionuclides are rapidly released from the cell after internalization and catabolism, while others are retained intracellularly and, therefore, are called residualizing radionuclides.

The most widely used radionuclide for RIT is ^{131}I, which is available at low costs and easy to couple to MAbs. The low β-energy and particle range of up to 2 mm, makes ^{131}I particularly well suited for treatment of small tumors. The γ-emission of ^{131}I, however, has a relative high energy of 364 keV, which limits its imaging quality for a gamma camera. The physical half-life of ^{131}I is long (8 days). In RIT studies that use ^{131}I as the therapeutic radionuclide, often an imaging study (also called pretherapy or scouting study) with a tracer dose of ^{131}I is performed in advance to confirm tumor targeting and to predict dosimetry for the individual patient. Alternatively, ^{123}I can be used as it has a more suitable γ-emission of 159 keV and a relative short half-life. A limitation for the use of ^{131}I in RIT is the occurence of dehalogenation, which requires the blocking of the thyroid with potassium iodide to prevent uptake of ^{131}I. Furthermore, during treatment extensive safety measures are required to limit the radiation dose to medical personnel, relatives, and environment caused by the accompanying γ-emission. Another therapeutic iodine isotope that has been used in clinical RIT is ^{125}I. This radionuclide has a long physical half-life of 60 days and produces low-energy Auger electrons. Due to the short path length of the electrons, less than a cell diameter, the radionuclide needs to be internalized in order to damage the nuclear DNA (*56*).

Another β-emitting radionuclide which has been extensively used in clinical RIT studies is ^{90}Y. The physical half-life of ^{90}Y is 64 hours and its decay is purely by β-emission, and, therefore, requires less extensive safety measures. Its high β-energy and particle range of up to 12 mm makes ^{90}Y particularly well suited for irradiation of larger tumors (*57*). Since ^{90}Y is a residualizing label, i.e. has a long residence time in the tumor cell after internalization, it can deliver a relatively large radiation dose. In RIT trials, it is customary to use ^{111}In as a γ-emitting surrogate for tracing the biodistribution of ^{90}Y.

Based on its physical properties, rhenium-186 (^{186}Re) might be another suitable candidate for RIT. Almost all decay (91 %) is by therapeutic β-emission with

an ideal energy and path length (5 mm) for irradiation of small to medium size tumors (*54,58,59*). The physical half-life of ^{186}Re is 3.7 days, which is compatible with the circulating half-life of intact MAbs. Moreover, ^{186}Re has a modest (9 %) part of low-energy γ-emission, which has excellent imaging properties, and can be used for confirmation of tumor targeting as well as for dosimetric purposes (*60*). For imaging in a scouting study prior to ^{186}Re-RIT, the pure γ-emitter technetium-99m (^{99m}Tc) might even be a better candidate radionuclide as it has similar chemical properties as ^{186}Re and delivers less radiation burden to the patient. Therapy with ^{186}Re requires acceptable radiation safety measures for both patients and medical personnel, and, therefore, outpatient-based treatment is a realistic option for its future application. Another rhenium isotope, ^{188}Re, has a relative short half-life for RIT with an intact MAb, while its high β-energy and particle range (11 mm) are in favor for irradiation of larger tumors. Clinical experience with ^{188}Re-labeled MAbs is limited.

Table 3. Main characteristics of β-emitting radionuclides used in clinical RIT studies.

Radionuclide	Half-life	Main β-energies		Main γ-energies		Maximum particle range
	(hours)	(keV)[1]	(%)[2]	(keV)	(%)	(mm)
Iodine-131	192	333	7	364	82	2.0
		606	89			
Yttrium-90	64	2284	100	--		12.0
Rhenium-186	89	939	21	137	10	5.0
		1077	71			
Rhenium-188	17	1965	25	155	15	11.0
		2120	71			
Copper-67	62	390	57	93	16	
		482	22	185	49	1.8
		575	20			
Lutetium-177	161	176	12	113	7	
		384	9	208	11	1.5
		497	79			

[1] Maximum β-energy, [2] Probability per decay.

Other suitable β-emitting radionuclides for RIT have been evaluated in few studies, among which are copper-67 (^{67}Cu) and lutetium-177 (^{177}Lu). Broad scale application of ^{67}Cu is still hampered by the lack of procedures for consistent production at high specific activities (*61*). ^{177}Lu is similar in chemistry to ^{90}Y, but has a considerable shorter maximum particle range of 1.5 mm, making it more suitable for treatment of small tumors.

RIT with α-emitting radionuclides might be suitable for eradication of circulating tumor cells and micrometastases or minimal residual disease, due to their short path-length of 1-10 cell diameters and high linear energy transfer. Bismuth-213 (^{213}Bi) with a physical half-life of 0.77 hours, bismuth-212 (^{212}Bi) with a half-life of 1 hour, astatine-211 (^{211}At) with a physical half-life of 7.2 hours, and actinium-225 (^{225}Ac) with a physical half-life of 10 days have been indicated to be suitable for RIT (*62-64*). The short physical half-life of ^{212}Bi, ^{213}Bi and ^{211}At, however, makes them less suitable for labeling of intact MAbs.

3.2 Dosimetry of radioimmunotherapy

Dosimetry is the study of energy deposition of radiation in tissue. Knowledge of dosimetry in tumor and normal tissues after administration of a certain amount of radiolabeled MAb is essential for an appropriate planning of RIT. Moreover, dosimetry can provide a better understanding of tumor dose-response relationships and the assessment of the toxicity to normal tissues, like the bone marrow. Radiation absorbed dose estimates can either be measured directly using mini-thermoluminescence dosimeters or calculated from biodistribution data obtained from biopsies or serial gamma camera imaging. Sinve decades many sophisticated models have been developed to address the problem of radiation dose calculation, such as the medical internal radiation dose (MIRD) formalism (*65*) and Monte Carlo techniques (*66-68*).

Most knowledge about tolerance and sensitivity of normal organs and tumors to radiation is derived from external beam irradiation studies. Tolerance doses to external beam irradiation for normal organs are well described and used in treatment planning (*69*). These data cannot be simply extrapolated to RIT, since there is a noticeable difference between both types of irradiation. External beam irradiation is delivered at a high-dose rate of usually 60 Gy/h, whereas RIT irradiation is delivered at low-dose rates, ranging from 0.1 to 0.2 Gy/h (*70,71*). Moreover, for RIT it has been

more difficult to establish a dose-response relationship than for conventional external beam irradiation, since the dose calculations for RIT are subject to more uncertainties, for example as a consequence of much more heterogeneous dose delivery (*72*). RIT, however, has shown to be able to generate clinical responses at relatively low tumor radiation dose estimates as compared to conventional external beam irradiation. On the other hand, the estimated tumor doses show large variation between individuals within a RIT study as well as between different RIT studies. Much effort has been put in the individualization of RIT dose planning, in order to increase its accuracy and to allow the identification of patients who will benefit from RIT, but also to identify patients who might experience toxicity to normal organs as a result of RIT. As indicated previously, for this purpose, often a pretherapy or scouting procedure has been explored, to obtain imaging and dosimetry data. This can be done with the same radionuclide as planned for RIT like in studies with ^{131}I-labeled MAbs. In this way, patients with non-Hodgkin's lymphoma have been treated with ^{131}I-labeled tositumomab at individualized therapeutic doses determined by a dosimetric study prior to RIT (*73*). The pretherapy study can also be performed with either a different or a chemically related radionuclide (matched pair). This has been done in ^{90}Y-RIT studies with ^{111}In-labeled MAbs for treatment planning (*74-78*), but also in RIT with ^{186}Re and ^{188}Re-labeled MAbs, where a scouting procedure with a ^{99m}Tc-labeled MAb was performed (*79-81*). From the pretherapy study, the radioactivity dose that results in a specific radiation absorbed dose to a particular organ or tumor can be estimated. Thus, the pretherapy study is a guide to the maximum amount of radioactivity that can be safely administered (*82*).

The most important dose limiting toxicity of RIT is bone marrow suppression, with a radiation dose limit to the bone marrow of 150-200 cGy. Radiation doses higher than 200 cGy usually lead to unacceptable toxicity, in general thrombocytopenia and leucocytopenia. Prediction of bone marrow toxicity with dosimetry, therefore, might improve the safety and efficacy of clinical RIT. Serial blood sampling in order to calculate the cumulated radioactivity in blood, together with the contribution of the whole body to the bone marrow dose, is currently accepted as the most accurate way to perform bone marrow dosimetry (*83,84*). With this approach, it is assumed that the bone marrow radioactivity levels are proportional to the blood activity levels (*85*). Although several studies have shown a good correlation between the bone marrow absorbed dose and the development of myelotoxicity, the blood-based bone marrow absorbed dose is not always a better predictor of myelotoxicity as compared to, for example, the total administered radioactivity dose or the whole body absorbed dose (*86-89*). Other, non-invasive approaches for bone marrow dosimetry are based on serial gamma camera imaging,

where the bone marrow dose is calculated using quantification of the cumulated radioactivity in the sacral and/or lumar spine area. Although this method in general does not show a better correlation with the development of myelotoxicity, it might be of value for accurate dosimetry in patients with bone marrow targeting as a result of tumor involvement or aselective trapping of the radionuclide (*90-92*).

3.3 Radioimmunotherapy and hematological malignancies

In the last decade, RIT was proven to be particularly effective in the treatment of hematological malignancies, especially non-Hodgkin's lymphomas (NHL). High complete response rates and increased duration of response have been observed in several phase II and III trials with radiolabeled anti-CD20 MAbs like ^{90}Y-ibritumomab tiuxetan (*93*) and ^{131}I-tositumomab (*73,76,94,95*), in patients with NHL refractory to chemotherapy. The results of these studies, as well as of RIT studies with other MAbs in patients with hematogical malignancies, have been reviewed by several authors (*96-100*). The success of ^{90}Y-ibritumomab tiuxetan and ^{131}I-tositumomab is thought to be due to several factors. Both conjugates make use of an anti-CD20 MAb, which demonstrates intrinsic activity consisting of ADCC, CDC, and apoptosis induction upon binding to the target cell. These MAbs are particularly effective because of the good accessibility of lymphoma tumors and cells for MAbs and the high and homogeneous expression of the CD20 target antigen. A next dimension was added by coupling radionuclides to these MAbs, because of the intrinsic radiosensitivity of lymphoma. Enhanced efficacy of radiolabeled MAbs for treatment of NHL was demonstrated by a recent randomized trial in which RIT using ^{90}Y-ibritumomab tiuxetan in combination with unlabeled cMAb rituximab (Zevalin™ protocol (*101*)) was compared with immunotherapy with cMAb rituximab alone (Rituxan™). Ibritumomab is the murine form from which chimeric rituximab has been constructed. In this trial the overall response rate was in favor of ^{90}Y-ibritumomab tiuxetan: 80 % versus 56 %, respectively (*75*). In addition, ^{90}Y-ibritumomab tiuxetan treatment resulted in an overall response rate of 74 % in rituximab-refractory patients (*102*).

3.4 Radioimmunotherapy and solid tumors

Most RIT studies in patients with solid tumors have been performed with a single intravenous administration of ^{131}I- or ^{90}Y-labeled MAbs in phase I and II trials. An overview of these studies is given in Table 4. Other routes of administration for RIT are intralesional, intrathecal, intra-arterial, intravesical, and intraperitoneal. These

techniques for locoregional treatment have been explored to a lesser extent than the intravenous route, and in most cases for selected tumor types.

Phase I and II studies, in general, have been conducted in a limited number of patients with recurrent or metastatic disease, after earlier standard treatment. These patients have been treated at escalating radiation dose steps, in order to determine the maximum tolerated dose (MTD) and the dose limiting toxicity (DLT) organ. This is the primary goal of phase I trials, which implies that most patients are treated at suboptimal radiation dose levels.

Most experience in RIT of solid tumors has been acquired from studies with gastrointestinal and colorectal cancer patients. These trials have been performed mainly with ^{131}I-labeled MAbs, directed against TAG-72 and carcinoembryonic antigen (CEA) (*34,105,107-110,115-117*). Murine MAb CC49, directed against the TAG-72 antigen, has been evaluated when labeled with ^{131}I, ^{177}Lu, and ^{90}Y. Immunogenicity of mMAb CC49 was relatively high, with development of HAMA in 75 to 100 % of the patients. Clinical response rates ranged from 17 to 25 % (*116,117,119,127*). The anti-CEA mMAb NP-4, has shown high tumor-absorbed doses in studies with ^{131}I-NP-4 F(ab')$_2$ and ^{131}I-NP-4 IgG, up to 12.9 and 218 cGy/mCi, and clinical responses in 46 and 34 % of the patients, respectively. Development of HAMA, however, was observed in 83 and 94 % of the patients (*34,107*). ^{188}Re-mMAb MN-14, also directed against the CEA-antigen, elicited HAMA responses in 80 % of the patients, and no clinical responses were observed (*121*). Its humanized counterpart (hMAb MN-14) was evaluated when labeled with ^{131}I and demonstrated high tumor-absorbed doses of 24.2 ± 22.6 cGy/mCi, but no clinical reponses in patients with mostly advanced and large metastatic lesions (*109*). More encouraging results were observed for ^{131}I-hMAb MN-14 in patients with small-volume disease of metastatic colorectal cancer. A total of 30 patients were treated with 60 mCi/m^2, and the overall response rate was 58 % in 19 evaluable patients (*108*). Another anti-CEA MAb, ^{186}Re-NR-CO-02 F(ab')$_2$, showed tumor-absorbed doses up to 18.6 cGy/mCi, and responses in 39 % of the patients (*120*). ^{131}I-mMAb A5B7 F(ab')$_2$ and intact IgG, also directed against the CEA antigen, showed low tumor-absorbed doses of 1.1 to 1.5 cGy/mCi, and clinical responses in 10-11 % of the patients (*105*). Further trials in patients with colorectal cancer have been carried out with MAbs directed against other antigens or pancarcinoma MAbs. ^{131}I-mMAb A33 showed development of HAMA in 100 % of the patients, and a 15 % responses rate (*103*). A mixed response in one patient and stabilization of disease in 12 out of 20 evaluable patients were observed for ^{125}I-mMAb A33 (*128*). Pancarcinoma antibodies mMAb NR-LU-10 and its chimeric counterpart NR-LU-13 showed HAMA and HACA development in 100 % and 75 % of the patients, respectively. No objective

clinical responses were seen in studies with ^{186}Re-labeled mMAb NR-LU-10 and cMAb NR-LU-13 in patients with colorectal, lung, and ovarian cancer (*42,120*).

In breast cancer patients, phase I RIT studies have been performed using MAbs directed against the breast mucine MUC-1, and the L6, Lewis^{y}, and TAG-72 antigens (*77,104,119,122,123*). High tumor doses up to 3700 cGy were achieved with ^{131}I-labeled cMAb L6, showing partial responses in 5 out of 10 patients treated (*104*). A study with ^{90}Y-labeled mMAb BrE-3, directed against the MUC-1 antigen, showed tumor doses ranging from 39 to 167 cGy, and only observation of mixed responses (*123*).

Patients with medullary thyroid carcinoma have been treated with anti-CEA MAbs NP-4 and MN-14 F(ab')$_2$, labeled with ^{131}I (*34,106,107*). ^{131}I-MN-14 F(ab')$_2$ elicited HAMA responses in 53 % of the patients, but high tumor-absorbed doses of 23.7 ± 27.5 cGy/mCi were observed, with stabilization of disease in 92 % of the patients (*106*). ^{90}Y-cMAb T84.66, a pancarcinoma MAb, was also evaluated in patients with medullary thyroid carcinoma, and showed clinical responses in 23 % of the patients, and an immune reponse in 59 % of the patients (*126*).

RIT in patients with ovarian cancer has been evaluated for intravenous and intraperitoneal administration. Studies on intravenous administration have shown some responses and stabilization of disease (*112,120,129*). No immunogenicity was observed in 3 patients treated with ^{131}I-cMAb MOv-18, and a mean tumor-absorbed dose of 27 cGy/mCi was observed (*112*). A mean tumor-absorbed dose of 8.0 cGy/mCi was found for ^{131}I-mMAb MN-14, but immunogenicity was 100 % (*111*). ^{186}Re-mMAb NR-LU-10, a pancarcinoma MAb, was evaluated in patients with ovarian cancer and other malignancies, and showed no clinical responses and 100 % development of HAMA (*120*).

Table 4. Results of clinical phase I/II radioimmunotherapy studies in patients with solid tumors. Conjugates contained β-emitting radionuclides and were administered intravenously.

Radio-nuclide	Target Antigen	Antibody	Type Antibody	Disease	Immuno genicity (%)	Injected Dose Range (mCi)	Blood Half Life[a] (h)	BM Dose Range (cGy)	BM Dose (cGy/mCi)	Tumor Dose Range (cGy)	Tumor Dose (cGy/mCi)	Response	Overall Response (%)	
I-131	A33	A33	murine	CRC	100	30-94 [b]						3/20 MR	15	(*103*)
I-131	L6	L6	chimeric	BRC	80	20-70 [b]				120-3700		5/10 PR	50	(*104*)
I-131	CEA	A5B7	$F(ab')_2$	CRC		82-148	38				1.5 ± 1.9	1/9 CR	11	(*105*)
I-131	CEA	MN-14	$F(ab')_2$	MTC	53	99-206	20	140-220		202-18268	23.7 ± 27.5	11/12 SD	92	(*106*)
I-131	CEA	NP-4	$F(ab')_2$	CRC, NSCLC, PA, MTC	83	68-254	15	55-450		511-6476	12.9	6/13 SD	46	(*107*)
I-131	CEA	MN-14	humanized	CRC		60 [b]		180 ± 80	3.0 ± 1.3			3/19 PR 8/19 MR	58	(*108*)
I-131	CEA	MN-14	humanized	GI, CRC		58-107			2.2 ± 2.4		24.2 ± 22.6	0/17	0	(*109*)
I-131	CEA	A5B7	murine	CRC		26-74	29				1.1 ± 2.1	1/10	10	(*105*)
I-131	CEA	F023C5	murine	CRC		50-80 [b]			0.54		185 (max)	1/10 CR 2/10 PR	30	(*110*)
I-131	CEA	MN-14	murine	OV	100	52-91	39	41-249	2.3 ± 0.7		8.0 ± 5.8	1/14 CR 1/14 MR	14	(*111*)
I-131	CEA	NP-4	murine	CRC, NSCLC, PA, MTC, BRC	94	44-268	21-36	45-706	3.0 ± 1.5		2-218	1/35 PR 4/35 MR 7/35 SD	34	(*34*)
I-131	folate	MOv-18	chimeric	OV	0	75-82		187-243	2.8 ± 0.3	600-3800	27.5 ± 22	3/3 SD	100	(*112*)
I-131	G250	G250	chimeric	RE	8	82-150	69 (β)					1/8 SD 1/8 PR	25	(*113*)
I-131	G250	G250	murine	RE	100	50-200	22					17/33 SD	52	(*114*)

I-131	TAG-72	B72.3	chimeric	CRC	58	34-67					2.3-5.9	4/12 SD	33	(*115*)
I-131	TAG-72	CC49	murine	CRC	92	75 [b]		60-117		19-667		3/15 SD	20	(*116*)
I-131	TAG-72	CC49	murine	CRC	100	45-228	39					6/24 SD	25	(*117*)
I-131	TAG-72	CC49	murine	PR	100	75 [b]				208-1083	3.4 ± 3.1	10/14	71 [c]	(*118*)
Lu-177	TAG-72	CC49	murine	CRC, NSCLC, BRC	100	16-45	67 (β)		4.0-5.0			2/9 SD	22	(*119*)
Re-186	CEA	NR-CO-02	$F(ab')_2$	NSCLC, CRC, GI	86	25-336	14		0.4 ± 0.1		0.4-18.6	1/31 PR 11/31 SD	39	(*120*)
Re-186	pan	NR-LU-10	murine	NSCLC, CRC, OV, RE	100	45-260	26		0.6 ± 0.2		0.4-17.7	0/15	0	(*120*)
Re-186	pan	NR-LU-13	chimeric	CRC, NSCLC, BRC, GI	75	42-129	35		1.3 ± 0.3		0.9-7.5	2/9 SD	22	(*42*)
Re-188	CEA	MN-14	murine	CRC	80	21-161	8		3.6 ± 1.6		16.2 ± 10.7	0/11	0	(*121*)
Y-90	$Lewis^y$	B3	murine	GI, BRC, NSCLC	100	5-25	77 (β)		4.8 ± 1.6		7.7-65.1	6/24 SD	25	(*122*)
Y-90	MUC-1	BrE-3	murine	BRC	83	9-17	87 (β)			442-1887	39-167	3/6 MR	50	(*123*)
Y-90	adeno	m-170	murine	PR	70	5-20 [b]		59-327	4.1-8.9		10-93	7/17 SD	41 [c]	(*124*)
Y-90	7^E11-C3.5	CYT-356	murine	PR	8	3-29	48				16.2 ± 21.9	0/10	0	(*125*)
Y-90	pan	T84.66	chimeric	CRC, NSCLC, MTC	59	8-43	-	5.3-171	0.5-4.6	66-1670	8.7-52.2	3/22 SD 2/22 MR	23	(*126*)
Y-90	TAG-72	CC49	murine	GI	75	17-38	47 (β)	50-120	2.5	180-3000	35	2/12 SD	17	(*127*)

[a] Monoexponential blood half-life; in some cases only the $T_{1/2}$ β is provided in the study, which is indicated (β).
[b] Injected dose range in mCi/m^2. [c] Only relief of pain was observed as clinical response.
Abbreviations: BM, bone marrow; CRC, colorectal cancer; BRC, breast cancer; MTC, medullary thyroid cancer; NSCLC, non-small cell lung cancer; GI, gastrointestinal cancer; PA, pancreatic cancer; OV, ovarian cancer; RE, renal cancer; PR, prostate cancer; CEA, carcinoembryonic antigen; MUC-1, breast mucin; CR, complete response; PR, partial response; SD, stable disease; MR, mixed or minor response; pan, pancarcinoma.

RIT was also evaluated in patients with prostate cancer with ^{131}I-mMAb CC49, ^{90}Y-mMAb CYT-356, and ^{90}Y-mMAb m-170. The only clinical responses observed comprised relief of pain, and development of HAMA occurred in 100 and 70 % of the patients for mMAb CC49 and m-170, respectively, and in 8% of the patients treated with mMAb CYT-356 (*118,124,125,130*). Modest anti-tumor effects were observed for ^{131}I-mMAb CC49, whengiven one week after administration of interferon-γ for upregulation of the TAG-72 target antigen (*130*). No clinical responses were observed in prostate cancer patients treated with ^{90}Y-mMAb CYT-356 (*125*).

Phase I/II trials with murine and chimeric MAb G250 have been conducted in patients with renal cell carcinoma, leading to stabilization of disease in 17 of 33 patients treated with ^{131}I-mMAb G250 (*114*). Immunogenicity was 100 % for mMAb G250, whereas this was reduced to 8 % for its chimeric counterpart (*113*). Moreover, the blood half-life of cMAb G250 was considerably prolonged as compared to mMAb G250 (*113*).

Besides intravenous also intraperitoneal RIT has been studied in ovarian cancer patients. This might be a more effective approach, especially in a small-volume or residual disease setting. Complete and partial responses, as well as prolonged survival were found in several intraperitoneal RIT studies with ^{131}I, ^{90}Y, ^{186}Re and ^{177}Lu-labeled MAbs (*131-134*). A multicentre, randomized, prospective phase III study of adjuvant therapy with anti MUC-1 MAb Pemtumomab (HMFG1), labeled with ^{90}Y, is currently conducted in patients with ovarian cancer. Evidence for a marked long-term survival benefit in patients receiving intraperitoneal ^{90}Y-Pemtumomab when in remission after surgery and chemotherapy has been observed in earlier phase II trials (*135,136*).

Intrathecal RIT has been given to patients with meningeal spread of primitive neurectodermal tumors, medulloblastoma, and leukemia. An advantage as compared to external beam irradiation, where the administered dose is limited by the radiation to the normal brain tissue, is the local application in the cerebrospinal fluid. For this group of patients, intrathecal RIT bypasses the problem of poor access to tumor antigens, which is the case for intravenously administered MAbs. Stabilization of disease was observed in 42 % of patients with leptomeningeal metastases of glioblastoma multiforme after treatment with ^{131}I-mMAb 81C6 (anti-tenascin) (*137*).

Intralesional RIT has been mainly applied for the treatment of malignant gliomas, with MAbs directed against the tenascin antigen, labeled with ^{131}I or ^{90}Y. Since malignant gliomas tend to spread by local invasion rather than by metastases, intralesional RIT might be an interesting approach for this type of cancer. Several studies with intralesional RIT have shown delivery of substantially higher radiation doses of 300 and 600 Gy for ^{131}I- and ^{90}Y-labeled MAbs than with other routes of administration (*138*). Direct injection of ^{131}I-mMAb 81C6 into surgically created resection cavities in patients with primary malignant gliomas resulted in an average absorbed dose of 41 Gy to the surgical cavity (*139*). Improved survival and high response rates have been observed in several studies with direct injection of radiolabeled

MAbs in patients with malignant gliomas (*138-140*). Intra-arterial and intravenous RIT has also been explored in patients with malignant gliomas with ^{125}I-labeled anti-epidermal growth factor receptor MAb 425, showing improved survival in both groups (*141,142*).

Intravesical RIT with ^{67}Cu-MAb C595 and ^{125}I-HMFG1, which are both directed against the MUC-1 antigen, has been explored for treatment of superficial urinary bladder cancer in biodistribution studies (*143,144*). To date, however, no clinical phase I RIT studies have been carried out.

Despite the encouraging results of RIT in patients with hematogical malignancies, such results have not been achieved as yet for solid tumors. In the majority of studies summarized in Table 4, tumor-absorbed dose estimates have been at most 20 to 30 Gy, and most clinical responses observed are stabilization of disease and minor or mixed responses, often of a short duration. Bone marrow toxicity has been dose limiting in all studies. However, encouraging results have been observed in patients with small tumor lesions or patients with (minimal) residual disease (*34,110,135,136,145*) and the first phase III trials in patients with minimal residual cancer of solid tumors have started over the last years (Table 2).

4. Strategies to improve radioimmunotherapy

The opportunities to enhance the therapeutic efficacy of RIT in solid tumors have to be based on improvement of the ratio between tumor and bone marrow absorbed dose. Strategies to increase the tumor-absorbed dose and/or to decrease bone marrow toxicity have been explored by various approaches. Factors influencing bone marrow toxicity are the radiation absorbed dose to the bone marrow, the baseline platelet or white blood cell count, the presence of bone or bone marrow metastases, and prior chemotherapy before RIT (*87,146-150*). Strategies to circumvent bone marrow toxicity have been explored with the use of autologous blood or bone marrow progenitor cell transplantation. These techniques include bone marrow transplantation and leucapheresis. Both have been successfully applied in RIT of patients with hematological malignancies, especially in patients with non-Hodgkin's lymphoma. Increased complete response rates of longer duration and prolonged survival have been achieved with RIT in patients with non-Hodgkin's lymphoma (*94,95,151-155*). In these studies higher radiation doses to tumor burden were delivered, while bone marrow toxicity was limited. Secondary dose limiting toxicities of normal organs were gastrointestinal and cardiopulmonary toxicity. RIT supported by autologous blood stem cell transplantation has been performed in patients with solid tumors, but only in a few studies. Table 5 summarizes results of phase I and II trials with autologous blood stem cell transplantation. Most studies have been conducted in patients with breast cancer using ^{131}I- or ^{90}Y-labeled MAbs (*156-160*),

but also in patients with colorectal (*161,162*), gastrointestinal (*163*), and medullary thyroid cancer (*164*). Substantially higher tumor-absorbed doses ranging from 10 to 70 Gy were achieved in these studies, leading to partial responses and stabilization of disease in a considerable number of patients.

Another approach to increase the tumor to bone marrow ratio is pretargeted RIT. In this technique, the radionuclide is administered separately from the tumor-targeting MAb. The aim of pretargeted RIT is to obtain higher and more selective accumulation of radioactivity in tumor, and to reduce toxicity resulting from circulating radiation in blood. It is a multistep procedure, where at first a non-radiolabeled MAb that targets the tumor antigen, coupled to streptavidin or avidin, is administered. A so-called clearing agent is given to achieve complete removal of this first MAb from the circulation. Radiolabeled biotin, with an extremely strong affinity for streptavidin and avidin, is then administered. Several modification of this biotin-streptavidin principle have been developed and have been reviewed recently (*165*). Evidence for higher tumor-absorbed doses and higher tumor to bone marrow ratios was observed in preclinical and clinical biodistribution studies with the biotin-streptavidin technique (*166-168*).

A different technique of pretargeted RIT involves the use of bispecific MAbs. These have mostly been modified with recombinant DNA techniques to allow targeting of two different antigens, one at the tumor site, and the other to a radiolabeled bivalent hapten. After the bispecific MAb has accumulated in the tumor, the radiolabeled bivalent hapten is administered. The latter is able to bind selectively to the second arms of two bispecific MAbs. This double binding at the tumor site is a more avid binding than the single binding with circulation bispecific MAb. Therefore, circulation bispecific MAb does not disturb tumor targeting with the bivalent hapten, and no clearance of the bispecific MAbs from the circulation is necessary with this variant of pretargeted RIT. This is an advantage as compared to RIT with the biotin-streptavidin technique. It simplifies the procedure, because a clearing step in pretargeted RIT requires careful dosing and timing of the clearing agent. Overdosing of the clearing agent, for example, may block the binding sites in the tumor and may thus reduce tumor targeting. Another disadvantage of the streptavidin-biotin pretargeted RIT is the presence of endogenous biotin and the development of human-anti-streptavidin responses, which can impede further administrations. Dosimetric analysis of pretargeted RIT with bispecific MAbs have shown evidence for high tumor-absorbed doses and high tumor to bone marrow ratios (*169,170*).

Table 5. Results of clinical Phase I/II radioimmunotherapy studies with autologous blood stem cell transplantation in patients with solid tumors.

Radio-nuclide	Target Antigen	Antibody	Disease	Injected Dose Range (mCi/m^2)	Bone Marrow Dose Range (cGy)	Tumor Dose Range (cGy)	Response	Overall Response (%)	Author
I-131	L6	L6	BRC	150	85-141	11,200	1/3 SD	33	(*156*)
I-131	CEA and TAG-72	COL-1 + CC49	CRC	75	`	393-1,327	4/14 SD	29	(*161*)
I-131	CEA	F6 $F(ab')_2$	CRC	87-300 mCi		30,000	1/9 PR 2/9 SD	33	(*162*)
I-131	TAG-72	CC49	GI	50-300	250-600	630-3,300	0/14	0	(*163*)
I-131	CEA	MN-14 $F(ab')_2$	MTC	232-486 mCi	450	1,104-73,640	1/12 PR 1/12 MR 10/12 SD	100	(*164*)
Y-90	mucin	M170	BRC	20-34	133-205	4600	2 PR 1 SD	100	(*158*)
Y-90	MUC-1	BrE-3	BRC	15-20		1,050-1,680	4/8 PR	50	(*159*)
Y-90	pancarcinoma	T84.66	BRC	15-22.5	76-177		1/6 PR 2/6 SD	50	(*160*)

Abbreviations: BRC, breast cancer; CRC, colorectal cancer; GI, gastrointestinal cancer; MTC, medullary thyroid cancer; PR, partial response; CR, complete response; SD, stable disease; MR, mixed or minor response.

To date, few clinical RIT studies have been performed with these two pretargeting RIT strategies. Encouraging results have been observed in biotin-streptavidin pretargeted RIT studies in patients with high-grade gliomas, where streptavidin conjugated anti-tenascin MAb BC4 was pretargeted for binding of ^{90}Y-labeled biotin. This resulted in tumor regression in 12/48 patients and prolonged survival in comparison with control patients (*171,172*). Murine MAb NR-LU-10 was evaluated in studies with patients with metastatic colon cancer (*173,174*). A five times higher radiation dose could be administered if pretargeted streptavidin-mMAb NR-LU-10 was used for binding of ^{90}Y-labeled biotin, resulting in tumor-absorbed dose of 4000 - 6000 cGy (*174*).

Evaluation of pretargeted RIT in NHL patients with ^{90}Y-labeled biotin pretargeted by anti-CD20 cMAb C2B8 revealed higher tumor to whole body ratios (38:1) and complete responses in phase I and II studies (*175,176*). Clinical phase I and II studies of pretargeted RIT with bispecific MAbs have been performed, demonstrating antitumor effects in patients with medullary thyroid cancer and non-small cell lung cancer (*177-179*). A chimeric bispecific MAb, composed of hMAb MN14 and mMAb 734, was evaluated in patients with CEA-expressing tumors, showing tumor doses ranging from 0.4 to 22.4 cGy/mCi (*179*). Bispecific MAb F6-m734 and ^{131}I-labeled bivalent hapten showed a tumor to bone marrow ratio of 29.6 ± 35.3, as well as relief of pain and minor tumor responses in 26 patients with medullary thyroid cancer (*177*). In non-small lung cancer, bispecific anti-CEA MAb mediated targeting of an ^{131}I-labeled bivalent hapten and resulted in partial responses in two patients and one stabilization of disease, with tumor doses ranging from 2.6 to 32.2 cGy/mCi (*178*).

Multiple administrations of antibodies or fractionated RIT can be performed to increase the tumor-absorbed dose, but is often hampered by the immunogenicity of MAbs. Development of chimeric and humanized MAbs have allowed multiple administration treatment regimens and higher total tumor-absorbed doses (*104,180,181*). Administration of immunosuppressive agents like cyclosporin A has also been explored to suppress the development of HAMA, but could not completely avoid a HAMA response and can lead to toxicity in patients (*156,182,183*).

Another way to increase the tumor-absorbed dose is by combining RIT with external beam irradiation. This has been performed in patients with colorectal cancer with metastatic disease to the liver, where RIT was combined with external beam radiotherapy to the liver (*184*). Combined RIT and external beam radiotherapy has been proposed for HNSCC by Maraveyas *et al.*, who reconstructed a theoretical phantom of the larynx to obtain dosimetric data. They were able to calculate dose-absorbed fractions for different radionuclides coupled to mMAb HMFG1, and demonstrated an increased tumor to mucosa ratio if external beam radiotherapy was

combined with RIT (*185*). A disadvantage of the combined approach is that the synergistic effect can only be given to a limited area. Occult metastatic or minimal residual disease cannot be effectively treated in this way.

5. Monoclonal antibodies for radioimmunotherapy of HNSCC

In the past, at least 30 MAbs directed against HNSCC have been described in literature, as extensively reviewed by Börjesson *et al.*(*186*). These MAbs can be divided in three main categories. The first group of MAbs is predominantly reactive with squamous cell carcinoma and much less with other tumor types. A general restriction of this group of MAbs is their reactivity with normal squamous epithelia. A second group of MAbs, also known as pancarcinoma MAbs, are not only directed against HNSCC, but also against other tumor types. To this group belong e.g. MAbs directed against CEA, EGFR, and the epithelial cell adhesion molecule (EpCam). A general shortcoming of these MAbs is their heterogeneous binding pattern with HNSCC, and their reactivity with several normal tissues. Most recently, a third group of MAbs became available. These MAbs do not bind to tumor cells themselves but to stromal cells or components involved in angionesis. Their clinical development, however, is at its infancy. Also for this category of MAbs, cross-reactivity with normal tissues might form a limitation.

Many of the MAbs described in literature are poorly characterized with respect to their reactivity profile, and only a few have been administered to HNSCC patients for radioimmunoscintigraphy (RIS) and biodistribution assessment. What is more, none of these MAbs had been evaluated in clinical RIT studies at the time this PhD study was started. Despite promising preclinical results, it may appear at an early stage of clinical studies, that the Mabs studied are not suitable for RIT. It is also possible that a MAb, though not selective enough for RIT, holds promise for other therapeutic approaches. This latter seems to be the case with MAbs directed against EGFR.

Extensive clinical RIS and biodistribution studies have been conducted with the mMAbs E48, U36 and BIWA 1, which are preferentially reactive with squamous cell carcinomas. MAb E48 is directed against a HNSCC-associated antigen belonging to the human Ly-6 antigen family (Ly-6D), while the epitope recognized by U36 has been mapped to the v6 domain of CD44 (*187*). At a later stage a second mMAb directed against CD44v6 became available called BIWA1. This mMAb binds to a different epitope than MAb U36, and with a 35-fold higher affinity (*33*). From clinical trials it was learned that mMAbs E48, U36, and BIWA1 are equally well suited for

detection of antigen-positive primary tumors and lymph node metastases of HNSCC, while biodistribution appeared similar (*35,188,189*). Extensive immunohistochemical analyses revealed selective reactivity of mMAb E48 with only normal and malignant squamous and transitional epithelia, while mMAbs U36 and BIWA 1 appeared also reactive with a variety of adenocarcinomas such as of the breast, colon, lungs, and stomach (*190,191*). This broader expression of the CD44v6 target antigen made mMAbs U36 and BIWA 1 first choice for further development to clinical RIT. The restricted expression pattern of the MAb E48-defined antigen Ly-6D, however, made this antigen the better marker for detection of circulating squamous cells in blood and bone marrow by reverse transcriptase polymerase chain reaction (RT-PCR) (*192*). CD44v6 appeared unsuitable for the latter approach due to its minor expression on a small subset of white blood cells.

6. Selection of HNSCC patients for radioimmunotherapy

The future role for RIT in treatment of HNSCC is thought to be a systemic adjuvant therapy. Therefore, identification of patients at risk for development of recurrent disease is of major importance. As stated earlier, the most important prognostic indicator for relapse of HNSCC, either locoregionally or at distant sites, is the presence of metastatic spread to lymph nodes in the neck (*11-16,19*). Among strategies used for other solid tumors to select patients at risk for development of recurrent disease is detection of micrometastatic cells in tissue samples such as surgical margins, regional lymph nodes, and bone marrow of patients (*193,194*). These micrometastatic cells can be detected by immunocytochemistry and molecular methods, notably polymerase chain reaction (PCR). Both techniques mainly have used tissue specific marker antigens such as cytokeratins, since these are abundantly expressed in the majority of epithelial tumors and homogeneously among the cells of these tumors. The clinical relevance has been illustrated especially for breast and colorectal cancer patients, in whom the presence of micrometastases in regional lymph nodes or in bone marrow correlates with poor prognosis (*194-199*).

Detection of single tumor cells in bone marrow of HNSCC patients proved feasible with immunocytochemistry techniques using monoclonal antibodies directed against cytokeratins (*200,201*). The presence of these cells at the time of primary treatment appeared to indicate a higher risk for development of both local recurrences and distant metastases, as well as a shorter disease-free survival time (*200,202,203*). In lymph nodes of HNSCC patients with histologically tumor-negative lymph nodes, detection of micrometastatic cells using reverse transcriptase PCR (RT-PCR) on the

HNSCC-associated antigen E48 was associated with a distinctly poor cause-specific survival (*204*). This RT-PCR assay is also able to detect E48 RNA transcripts in blood and bone marrow, and showed evidence for the presence of micrometastatic cells in bone marrow in 35% of HNSCC patients, whereas non-cancer controls were all negative (*192*). The early detection of micrometastatic cells in bone marrow of HNSCC patients could contribute to a more accurate staging and identification of patients at risk for development of recurrent disease, particularly at distant sites.

7. Aim of the study

The primary aim of this thesis was to investigate the feasibility of RIT with CD44v6 targeting MAbs in HNSCC patients. Besides that, the value of E48 RT-PCR on bone marrow aspirates for detection of micrometastatic cells was determined in order to identify patients at risk for development of recurrent HNSCC. In chapter 2, the results of a phase I radiation dose-escalation study with ^{186}Re-cMAb U36 in HNSCC patients are described. Evaluation of a pretherapy study with ^{99m}Tc-cMAb U36 to predict the pharmacokinetics and myelotoxicity of ^{186}Re-cMAb U36 RIT is reported in chapter 3. In order to explore further radiation dose escalation with ^{186}Re-cMAb U36, a study with autologous peripheral blood stem cell support was performed (chapter 4). To deal with the problem of immunogenicity, the humanized anti-CD44v6 MAb (hMAb BIWA 4) was evaluated in a biodistribution study with patients treated for primary HNSCC, of which the results are described in chapter 5. Finally, chapter 6 evaluates the suitability of the E48 RT-PCR assay for detection of micrometastatic cells in bone marrow aspirates of patients treated for primary HNSCC to select patients who are at risk of developing recurrent disease, particularly at distant sites.

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Phase I therapy study of ^{186}Re-labeled chimeric monoclonal antibody U36 in patients with squamous cell carcinoma of the head and neck

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Abstract

A phase I therapy study was conducted to determine the safety, maximum tolerated dose (MTD), pharmacokinetics, dosimetry, immunogenicity, and therapeutic potential of ^{186}Re-labeled anti-CD44v6 chimeric monoclonal antibody U36 (cMAb U36) in patients with squamous cell carcinoma of the head and neck (HNSCC). The potential of a diagnostic study with ^{99m}Tc-cMAb U36 to predict the biodistribution of ^{186}Re-cMAb U36 was evaluated.

Thirteen patients with recurrent or metastatic HNSCC were given 20 mCi (750 MBq) ^{99m}Tc-labeled cMAb U36 (2 mg), followed one week later by a single dose of ^{186}Re-cMAb U36 (12 or 52 mg) in radiation dose escalating steps of 11, 27, and 41 mCi/m^2 (0.4, 1.0, and 1.5 GBq/m^2). After each administration planar and SPECT images were obtained, and pharmacokinetics and development of human-anti-murine as well as anti-chimeric MAb responses were determined. Radiation absorbed doses to tumor, bone marrow, and organs were calculated.

Administrations were well tolerated and excellent targeting of tumor lesions was seen in all patients. Dose limiting myelotoxicity (thrombocytopenia being most prominent) was the only toxicity observed, resulting in grade 4 myelotoxicity in two patients treated with 41 mCi/m^2. The MTD was established at 27 mCi/m^2, at which a transient grade 3 thrombocytopenia was seen in one patient. One patient showed stable disease for 6 mo after treatment at the MTD. The two patients with dose limiting myelotoxicity both showed a marked reduction in tumor size. The reduction was of short duration and, therefore, not considered an objective response. Tumor absorbed doses at MTD ranged from 3.0 to 18.1 Gy. Bone marrow doses ranged from 20 to 112 cGy (mean: 1.9 ± 0.6 cGy/mCi or 51 ± 16 cGy/GBq) and correlated with platelet nadir (r = 0.8, P < 0.01). Pharmacokinetics varied between patients treated at the same dose level, and were accurately predicted by the diagnostic procedure. Five patients experienced a human-anti-chimeric antibody response, one of which was a human-anti-mouse antibody response.

This study shows that ^{186}Re-cMAb U36 can be safely administered, with dose-limiting myelotoxicity at 41 mCi/m^2. The use of cMAb U36 instead of its murine counterpart did no decrease the induction of human antibody responses. The availability of a ^{99m}Tc-labeled diagnostic study that can predict the pharmacokinetics of ^{186}Re-cMAb U36 offers the possibility of using such a study for selection of a safe RIT dose.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) accounts for about 5% of all newly diagnosed malignancies in North-Western Europe and the United States and the world wide incidence is 500,000 cases each year (*1*). Despite improvements in locoregional treatment modalities by surgery and radiotherapy for stages III and IV (70%), the high failure rate, either locally or at distant sites, is still high. An effective adjuvant systemic treatment is therefore needed to improve survival in this patient group.

Among the novel approaches for selective treatment is the use of monoclonal antibodies (MAbs), conjugated with radionuclides. In the last decade, successful treatment with radioimmunotherapy (RIT) of hematological malignancies refractory to chemotherapy has been reported (*2,3*). However, for solid tumors such results have not yet been reached, although some studies have reported encouraging results with RIT of small metastatic lesions or as adjuvant treatment (*4-8*). To date, most clinical RIT studies have been performed with ^{131}I. However, the low percentage (33%) of therapeutic ß-emission of ^{131}I is suboptimal and its abundant γ-emission can cause unnecessary radiation burden for medical personnel and relatives, requiring extensive radiation safety measures. Rhenium-186 (^{186}Re) is considered to be a better candidate, since almost all decay (91%) is by therapeutic ß-emission with an ideal energy for eradicating minimal residual disease: a maximum ß-energy of 1.07 MeV, and 90% of it delivered within 1.8 mm of a point source (*9,10*). The 9% γ-emission has excellent imaging properties for scintigraphy and dosimetry, and results in minimal radiation exposure of medical personnel. Its physical half-life of 3.7 days is considered compatible with the time needed for intact MAbs to achieve optimal tumor to non-tumor ratios. To date, few studies have been performed with ^{186}Re-labeled MAbs (*11-13*).

Murine MAb U36 (mMAb U36) has been extensively studied in patients with primary HNSCC and lymph node metastases undergoing surgery (*14,15*). Tumor uptake values up to 43% of the injected dose per kg (%ID/kg) tumor were reached, with a mean value of 20.4 %ID/kg two days after administration (*14*). Immunohistochemistry has shown reactivity with 99% of primary HNSCC and a homogenous reactivity pattern with 96% of these tumors, making the antibody an ideal candidate for targeting nearly all HNSCC (*16*). The antigen recognized by MAb U36 is the v6 domain of the CD44 glycoprotein (*17*). The development of human-anti-mouse (HAMA) responses in patients treated with mMAb U36 could hamper its use for multiple treatment RIT, and a chimeric MAb U36 (cMAb U36) was therefore constructed.

This phase I radiation dose escalation trial with ^{186}Re-labeled cMAb U36 was performed on HNSCC patients to determine safety, dose limiting toxicity, and maximum tolerated dose. The pharmacokinetics, biodistribution, immunogenicity, imaging, dosimetry, and therapeutic effects were studied to evaluate its potential for adjuvant treatment of HNSCC. The feasibility of a scouting study with ^{99m}Tc-labeled cMAb U36, given one week prior to RIT to predict the biodistribution of cMAb U36, was assessed.

Materials and methods

Patients eligibility

Patients with clinical evidence of recurrent HNSCC, either locally or at distant sites, refractory to conventional therapy or for whom no curative options were available, were candidates. A histologically confirmed HNSCC in the past was required for inclusion. Other eligibility criteria were: age between 18-80 y, a Karnofsky performance status of at least 60, and a life expectancy of at least 3 mo. An interval of at least 6 wk from the last chemotherapy and/or radiotherapy was required, as well as good recovery after prior treatment. Patients were excluded if their white blood cell count was below 3,500/μL, platelets below 150,000/μL, serum creatinine concentration above 150 μmol/mL, or bilirubin level above 40 μmol/mL. Other exclusion criteria were pregnancy, administration of murine antibodies in the past, evidence of a life-threatening infection, allergic diathesis, or serious cardiac disease. The study was approved by the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands). All patients signed informed consent after thorough explanation of the study.

cMAb U36

MAb U36 (Centocor B.V., Leiden, The Netherlands) was generated by immunizing BALB/c mice with viable cells of the HNSCC cell line UM-SCC-22B (*18*). The antigen recognized by MAb U36 is a 200 kDa glycoprotein located on the outer cell surface. Characterization of the target antigen by cDNA cloning and sequence analysis revealed that the antigen is identical to keratinocyte-specific CD44 splice variant epican (*17*). By screening overlapping synthetic peptides the epitope appeared to be located in the v6 domain of CD44 (*17*). Expression of the v6-containing CD44 variants has been observed in a variety of carcinomas, including head and neck, lung, skin, espohagus, and cervix, but also in adenocarcinomas of the

breast, colon, lung, and stomach (*19,20*). In patients with carcinoma of the breast and colon and with non-Hodgkin's lymphoma, a correlation has been shown between an overexpression of CD44v6 and reduced survival (*21-23*). The CD44v6 overexpression is thought to confer metastatic potential to tumor cells (*24*). Screening of normal tissues revealed expression in skin keratinocytes, breast and prostate myoepithelium, and bronchial epithelium (*18,20,25*).

In previous radioimmunoscintigraphy (RIS) studies, HAMA responses were observed in 37% of patients (*15*). Therefore, for reduction of immunogenicity, cMAb U36 was constructed, containing the murine-variable domains attached to the human γ1 heavy-chain and human κ light-chain constant regions by using recombinant DNA technology as described (*26*).

Radiolabeling and quality controls

All patients received both ^{99m}Tc-cMAb U36 and ^{186}Re-cMAb U36. The final quality of each batch of radiolabeled cMAb U36 was tested (*27*). For coupling of ^{99m}Tc and ^{186}Re to cMAb U36 the same chelate S-benzoyl-mercaptoacetyltriglycine (MAG3) was used as described previously (*27,28*). Radionuclides as well as the chelate were obtained from Mallinckrodt Medical, Petten, The Netherlands. The mean specific activity of ^{186}Re at the time of labeling was 358 ± 151 μCi/nmol (13.2 ± 5.6 MBq/nmol). In short, after synthesis of ^{99m}Tc-MAG3 or ^{186}Re-MAG3, an esterification with tetrafluorophenol (TFP) was performed. Subsequently, the ester was purified and conjugated to cMAb U36. Radiolabeled cMAb U36 was purified on a PD10 column with 0.9% NaCl as eluent for the ^{99m}Tc-cMAb U36 conjugates and 0.9% NaCl with 5 mg/mL ascorbic acid (pH 5) for the ^{186}Re-cMAb U36 conjugates. Finally, the conjugates were filter sterilized. The ^{186}Re-MAG3:cMAb U36 ratio was always lower than 4. The radiochemical purity of the conjugates was more than 93% (mean, 97%), as assessed by high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) of the final product. Each radiolabeled cMAb U36 preparation was assayed for immunoreactivity by measuring binding to 0.1% paraformaldehyde fixed cells of the HNSCC cell line UM-SCC-11B as described before (*27*). The immunoreactive fraction of both ^{99m}Tc- and ^{186}Re-cMAb U36, as determined by a modified Lineweaver Burk plot, ranged from 77 to 100% (mean: 96%) for all preparations.

Prestudy screening and radiological assessment

The patients' history and physical condition were examined, and routine laboratory analyses were performed, including serum electrolytes, hepatic enzymes, thyroid and renal function, and urine. Complete blood cell and platelet counts, an

electrocardiogram, and chest radiograph were obtained. CT or MRI scans were obtained as previously described (*15*).

Tumor volume was assessed by an experienced radiologist drawing regions of interest (ROIs) on consecutive CT or MRI slices with the use of a VoxelQ system (Picker International Highlands Heights, OH), which enables 3-dimensional reconstruction and automated volume calculation. Volumes of tumor recurrences or metastases were determined in cubic centimeters and used for tumor dosimetry and evaluation of efficacy of RIT. Because of the use of a gantry tilt in some of the CT studies, 3-dimensional reconstruction and automatic volume calculation was not always possible. In these cases, the sum of individual ROI surfaces and slice thicknesses was used for tumor volume estimation, and corrected for the degree of gantry tilt.

Study design

All patients underwent a scouting study prior to RIT with 2 mg of ^{99m}Tc-cMAb U36 (20 mCi/750MBq) to evaluate whether such procedure could predict the biodistribution of cMAb U36. A complete pretreatment assessment was performed before this study and blood samples were taken for pharmacokinetic analyses. Irrespective of the outcome of the RIS study, RIT was given within one week in escalating dose steps of ^{186}Re-cMAb U36. Two patients (patients no. 1 and no. 2) received 12 mg cMAb U36, labeled with 11 mCi/m^2 of ^{186}Re. All other patients received 52 mg cMAb U36, labeled with escalating radiation doses. The dose levels were 11, 27, 41 mCi/m^2 (0.4, 1.0, 1.5 GBq/m^2), and were planned to rise with escalating steps of 14 mCi/m^2 (0.5 GBq/m^2). Vital functions (blood pressure, pulse rate, breathing rate, and temperature) were assessed before administration; after 20, 40, 60, 120, and 240 min; and at 21 h.

Safety

Patients were admitted for 21 h in a special treatment room at the Department of Nuclear Medicine. Thereafter, they stayed for an additional 3 days in a single room. Dose rates (in μSv/hr) were measured directly after administration, and after 21 and 72 h at distances of 50 and 100 cm, with a gamma radiation dose rate counter (Berthold LB 123, Ortec, Oak Ridge, TN). The patients were discharged 4 days after administration of ^{186}Re-cMAb U36, and the set of pretreatment laboratory analyses was repeated weekly for at least 6 wk. The severity of toxicities was graded according to the National Cancer Institute Common Toxicity Criteria. The maximum tolerated dose (MTD) was defined as the dose level at which a grade 4 hematological or grade 3 nonhematological toxicity developed in not more than 1 out of 6 patients. A complete response was defined as disappearance of all measurable disease by physical examination or radiographic criteria for a duration of at least 4 wk. A partial response was defined as a reduction of at least 50% in the sum of the products of the perpendicular diameters of all index lesions for at least 4 wk and no new lesions. Stable disease was defined as a 50% reduction or a 25% or smaller increase in the sum of the perpendicular diameter products and no new lesions. This state had to persist for a minimum of 3 mo. Progression was defined as an unequivocal increase in size (>25%) of any lesion or the appearance of a new lesion.

Imaging studies

The same acquisitions were made for RIS with ^{99m}Tc-cMAb U36 as for RIS with ^{186}Re-cMAb U36. In the ^{99m}Tc-RIS procedure, simultaneously acquired planar

anterior and posterior whole-body scans were obtained within 1 h after administration of the radioimmunoconjugate and after 21 h. For the ^{186}Re procedure, scans were obtained after 72 and 144 h and, if feasible, for patients up to 2 wk. For all scintigraphic studies, a large field-of-view gamma camera (Dual Head Genesys Imaging System, ADAC Laboratories, Milpitas, CA) equipped with low-energy high-resolution parallel-hole collimators and connected to a computer system (Pegasys, ADAC Laboratories, Milpitas, CA) was used. Use of the same collimator for ^{99m}Tc-cMAb U36 as for ^{186}Re-cMAb U36 was expected to result in good image resolution. The low percentage of high-energy gamma photons of ^{186}Re (0.05% > 600 KeV) was not expected to require the use of a medium-energy collimator, as was done in other studies (*29,30*). Camera quality-control measures were taken at each imaging time point. With the aliquot retained from the radioimmunoconjugate preparation for injection, a weighed dilution of the injected patient dose was prepared as standard and measured simultaneously during imaging on all whole-body scans. Lateral, anterior, and posterior planar images and SPECT images of the region of tumor recurrence, that is, the thorax or head and neck region, were acquired at 21 h for ^{99m}Tc-RIS and 21, 72, and 144 h after administration in case of ^{186}Re-RIS. Acquisition parameters for planar and SPECT imaging were as previously described (*14*). In short, studies were performed with 360 degrees rotation, 64 steps, and postfiltering with Hanning filter (cut-off at 1 cycle/cm). Imaging results were interpreted by an experienced examiner, who was unaware of the site of recurrence or the presence of distant metastases.

Pharmacokinetics

Blood samples were taken from the opposite antecubital vein at 5, 10, 30 min, and at 1, 2, 4, 16, and 21 h after injection of ^{99m}Tc- as well as ^{186}Re-cMAb U36. For ^{186}Re-cMAb U36, additional samples were taken at 72 and 144 h after injection. Whole blood and plasma samples were counted in a multiwell gamma counter (1470 Wizard, Wallac, Turku, Finland) and radioactivity levels were expressed as percentage of the injected dose per kg (%ID/kg). Corrections were made for background and decay; the %ID/kg was determined by comparison with an aliquot retained from the conjugate preparation for injection. For ^{186}Re-cMAb U36, an area under the time-activity concentration curve (AUC) to infinity was calculated using a curve-fitting program (Multifit, Groningen, The Netherlands) to generate monoexponential or biexponential clearance curves. To compare the pharmacokinetic behavior of both radioimmunoconjugates for each individual patient, AUCs from 0 to 25 h after injection were calculated. The biologic half-life was calculated as $t_{1/2}$, or $t_{1/2}\alpha$ and $t_{1/2}\beta$ for monoexponential or biexponential clearance, respectively, of the radioimmunoconjugate, depending on its best fit. HPLC was performed on plasma

samples to assess the percentages of radiolabeled free cMAb U36 (retention time: 15.9 ± 0.2 min) and complexed cMAb U36 (retention time: 11.1 ± 1.1 min), essentially as previously described (*27*).

Dosimetry

A biological whole-body half-life ($t_{1/2\ wb}$) was calculated using least square fitting of the curve for whole-body time *versus* %ID. Extrapolation was done from the latest time points to infinity. The conjugate view counting technique was used for quantification of activity in the organs, and the initial whole-body counts were regarded as representing 100% of the injected dose. On all whole-body scans, regions of interest (ROIs) were drawn around the following organs: liver, lungs, spleen, and left kidney. For dosimetry of the lungs, a smaller ROI was drawn in the right lung to reduce the contribution of scatter originating from the cardiac blood-pool and liver. Total lung counts were obtained by correction of the counts per pixel of the smaller ROI for the total lung size. A background ROI was drawn in the lower left quadrant of the abdomen, and 75% and 50% of the counts was used for background correction of activity in the spleen and kidney, respectively (*31*). For lung and liver, no background correction was performed. An effective attenuation factor was derived from phantom studies by Breitz *et al.*, who obtained fractions of counts transmitted to examine the attenuation for ^{186}Re (*29*). The background subtracted counts from each organ were multiplied by this attenuation factor. Correction for counting efficiency of the gamma camera was done using an imaged standard with ^{186}Re. The %ID was calculated for each organ at each imaging time point, and the effective AUC for the whole-body as well as for all organs was used to obtain residence times in a spreadsheet software program (Lotus 1-2-3, release 5, Lotus Development Corporation, Cambridge, MA). Residence times were implemented in MIRDOSE 3 software (Oak Ridge Associated Universities, Oak Ridge, TN), to obtain S values and the total absorbed radiation dose per injected dose (cGy/mCi and cGy/GBq) to organs and the whole-body.

Using the whole blood time-activity concentration curve for ^{186}Re-cMAb U36, AUCs to infinity were calculated for bone marrow dosimetry. Patient-specific bone marrow dosimetry was performed according to Shen *et al.* (*32*). This method also considers the contribution of other organs and the whole-body. The concentration of intact MAbs in bone marrow is regarded 0.2-0.4 times that in circulating blood, and this bone marrow-to-blood ratio was determined using the patients' hematocrit and bone marrow extracellular fluid fraction (*33*). A comparable clearance from blood and bone marrow was assumed, as well as a uniform distribution throughout the bone marrow.

A ROI for the tumor was drawn on the anterior whole-body scan made at 72 h after injection and then copied to anterior scans acquired at other time points. Another ROI was drawn for tumor background correction on the contralateral side of the neck. The relative tumor-to-neck diameter, as assessed with CT or MRI, was used for background correction according to Buijs *et al.* (*31*). Background-subtracted counts were corrected for attenuation depending on tumor depth derived from CT or MRI results and the effective attenuation factor (*29*). Tumor volume was measured to obtain S values for tumor nodules in MIRDOSE 3 software. The tumor absorbed dose estimates were obtained after implementation of the residence times using MIRDOSE 3 software. For each evaluable patient, the ratio of tumor absorbed dose to bone marrow was determined.

Human anti-cMAb U36 response

To evaluate the immunogenicity of cMAb U36, a human-anti-chimeric antibody (anti-isotypic, HACA) assay was performed. To evaluate whether there were responses against the murine part (anti-idiotypic, HAMA), a second assay was included. Human antibody response was tested in patients' sera before administration of ^{99m}Tc- and ^{186}Re-cMAb U36, and 1 and 6 wk after the start of the RIT. For the detection of HAMA, an enzyme-linked immunosorbent assay (ELISA) with mMAb U36 was used as described previously (*15*). In short, ELISA plates were coated with goat polyclonal anti-mouse IgG, followed by mMAb U36 solution. Prediluted patient serum samples were added, and for detection, rabbit polyclonal anti-human IgG conjugated with horseradish peroxidase (HRP) followed by *o*-phenylenediamine was used. The sensitivity of the HAMA assay was at a titer of 50. A HAMA titer of 3 times this limit (> 150) was arbitrarily considered positive.

The concentration of HACA was measured by coating a microtitre plate (Costar, Europe Ltd, Badhoevedorp, The Netherlands) with cMAb U36 IgG, 2 µg per well, in phosphate buffered saline (PBS), pH 7.2, and incubating overnight at room temperature. After the contents were discarded, the plate was blocked with 200 µL assay buffer per well (1% volume in volume newborn calf serum [BioWhittaker, Verviers, Belgium] in PBS with 0.02% Tween 20 [Sigma, Zwijndrecht, The Netherlands]), by incubating at 37° C for 1 h. The wells were then washed with 200 µL wash buffer per well (PBS with 0.05% Tween 20). Thereafter, 100-µL of standard dilutions of rabbit anti-human IgG (A0424 DAKO, Glostrup, Denmark) in assay buffer and diluted patients' serum (1:10 in assay buffer) were pipetted into the wells and incubated on an orbital shaker at 400 rpm for 1 h at room temperature. The rabbit anti-human IgG was used to construct a calibration curve. The wells were washed, and after the addition of 100 µL biotinylated cMAb U36 in assay buffer (1 µg per

well), the plate was incubated as in the previous step. After washing, the wells were incubated with 100 µL (10 ng per well) poly-HRP-conjugated streptavidin (CLB, Amsterdam, the Netherlands) in assay buffer for 20 min and 400 rpm at room temperature. The wells were then washed, and HRP activity was determined by adding 100 µL tetramethylbenzidine substrate solution per well, (100 mg/L tetramethylbenzidine in 0.1 mol/L sodium acetate and citric acid buffer [pH 4.0]) and incubated for 30 min in the dark at room temperature. The reaction was stopped with 50 µL 2N H_2SO_4 per well, and the absorption was measured at 450 nm in a microtiterplate reader. The sensitivity of the HACA assay was at a concentration of 0.2 mg/L. The intra-assay coefficient of variation (CV), as calculated from duplicate measurements in serum samples with test results greater than the detection limit, was 6.5%. A HACA test value three times this limit, > 0.6 mg/L, was arbitrarily considered positive.

Statistical analysis

All mean values reported represent arithmic means with corresponding SD. Associations between variables were calculated with SPSS 7.5 software (SPSS Inc., Chicago, IL) using Pearson correlation tests. Two-sided significance levels were calculated for all parameters, with $P < 0.05$ considered statistically significant.

Results

Thirteen patients (ten men, three women; age range 47-68 y; mean age 57 y) entered this study. Their characteristics are shown in Table 1.

Safety and non-hematological toxicity

All administrations of ^{99m}Tc- and ^{186}Re-cMAb U36 were well tolerated by patients and no signs of acute adverse events were observed. No relevant changes occurred in physical parameters during the first few days after administration, and no changes in blood parameters indicated toxicity of organs such as the liver or kidneys. Although in some patients accumulation of radioactivity in feces was visible by scintigraphy, no symptoms of gastrointestinal radiation toxicity were observed. Two weeks after ^{186}Re-cMAb U36 administration, bilateral erysipelas developed in the face of patient 2, and was effectively treated with antibiotics. Patient 5 died two days after administration of ^{186}Re-cMAb U36, most likely because of cardiopulmonary insufficiency or a myocardial infarction. In this patient, scintigraphy 21 h after administration of ^{99m}Tc-cMAb U36 showed relatively high uptake in the lungs, but this was not observed on images obtained after ^{186}Re-cMAb U36 injection. This

apparant difference was considered to be related to the difference in MAb doses used in RIS and RIT. No other signs indicating a possible relationship between antibody administration and death were noticed, but the possibility of a relationship could not be excluded.

Mean radiation dose rates at 1 m distance from the patients treated at the highest dose level were 3.2 μSv/h directly after injection, and 1.6 μSv/h at discharge 3 days after injection. These dose rates would result in cumulative doses far below the annual limit of 2 mSv considered acceptable for medical personnel and family members.

Table 1. Patient and Tumor Characteristics.

Patient	Sex	Age	Site of Disease	Prior Treatment	
no.		(y)		Radiotherapy	Chemotherapy
1	M	53	oropharynx	yes	MTX
2	F	54	posterior pharyngeal wall	yes	none
3	M	54	oropharynx	yes	none
4	M	47	oropharynx	no	none
5	M	56	piriform sinus left and right parapharyngeal area	yes	Cis+5-FU
6	M	66	larynx and cystic lesion neck (right side)	yes	Cis+Gem/ MTX
7	F	56	oropharynx, both sides	yes	none
8	M	58	hypopharynx	yes	none
9	M	67	neck recurrence, left side	yes	none
10	M	68	oropharynx, right axilla metastasis	yes	none
11	M	52	larynx	yes	none
12	M	52	floor of mouth/ submental recurrence	yes	Cis+Gem
13	F	59	esophagus	yes	none

Abbrevations: M, male; F, female; MTX, methotrexate; Cis, cisplatin; 5-FU, fluorouracil; Gem, gemcitabine.

Table 2. Absorbed Dose Estimates and Myelotoxicity.

Patient no.	Dose	Total dose administered	Absorbed dose, whole-body	Absorbed dose, bone marrow	Platelets nadir	Toxicity grade	WBC nadir	Toxicity grade	Granulocytes nadir	Toxicity grade
	(mCi/m^2)	(mCi)	(cGy/mCi)	(cGy)						
1	11	13	1.3	46	230	0	3.4	1	2.3	0
2	11	16	0.9	25*	NA	NA	NA	NA	NA	NA
3	11	19	1.2	30	200	0	6.1	0	5.2	0
4	11	19	1.3	20	285	0	5.7	0	4.1	0
5	27	43	0.8	73*	NA	NA	NA	NA	NA	NA
6	27	57	0.8	109	37	3	3.6	1	2.1	0
7	27	44	NA	91	206	0	5.3	0	4.5	0
8	27	46	1.4	77	97	1	4.7	0	3.7	0
9	27	46	1.1	110	190	0	7.6	0	6.2	0
10	27	48	1.1	100	126	0	4.9	0	3.4	0
11	41	80	1.0	104	118	0	4.3	0	3.2	0
12	41	59	0.3	107	24	4	0.6	4	0.1	4
13	41	58	1.1	112	22	4	2.3	2	1.8	1

Abbrevations: NA, not available; wbc, white blood cell count.*Patients 2 and 5 did not complete follow-up for evaluation of hematological toxicity and are therefore not included for analysis of correlation between the bone marrow dose and development of hematological toxicity.

Hematological toxicity

The bone marrow appeared to be the dose-limiting organ. Myelotoxicity consisted mainly of thrombocytopenia, with a nadir 4 wk after RIT. At the lowest dose level -11 mCi/m^2- no clinically relevant hematologic toxicity was seen, whereas at the next dose level - 27 mCi/m^2- only mild toxicity was observed (Table 2). In one patient at this dose level (patient 6) grade 3 thrombocytopenia and grade 1 leucocytopenia developed, and the patient recovered from both conditions within 1 wk. He had been treated 14 mo before RIT with two courses of cisplatin and gemcitabine, and had received a weekly dose of 60 mg methotrexate for 20 wk 6 mo before RIT. Of three patients treated with 41 mCi/m^2, grade 4 thrombocytopenia developed in two. One of these two patients (patient 13), who had never been treated with chemotherapy, showed recovery within 1 wk from a grade 4 thrombocytopenia and grade 2 leucocytopenia. The other patient (patient 12) had previously experienced myelotoxicity before as a result of 3 courses of cisplatin and gemcitabine, given 6 mo before entry in the RIT study, but hematological parameters had recovered to baseline. In that patient, grade 4 thrombocytopenia and leucocytopenia developed 4 wk after administration of ^{186}Re-cMAb U36, and he died from an opportunistic lung infection shortly thereafter. Autopsy confirmed bilateral bronchopneumonitis with signs of an adult respiratory distress syndrome resulting from pneumonia caused by a mixture of *S. Pneumoniae, E. Coli,* and *P. Aeruginosa*. Although the bone marrow was greatly aplastic, some erythropoietic activity was seen. On the basis of the results, 27 mCi/m^2 was regarded to be the MTD level.

Imaging

Whole-body scans obtained directly after administration of ^{186}Re-cMAb U36 showed mainly blood-pool activity, which was decreasing over time but clearly present up to 72 h after injection in most patients. Representative whole-body scans are shown in Figure 1. At later time intervals, a homogeneous distribution was observed in the lungs, liver, spleen, and kidneys, except for increased uptake at tumor sites. In general, no selective accumulation at nontumor sites was visible, except in feces and urinebladder. In one patient, a relatively high accumulation was seen in the liver at 21 h after injection. This patient (patient 2) also had a relative rapid clearance of ^{186}Re-cMAb U36 from the blood.

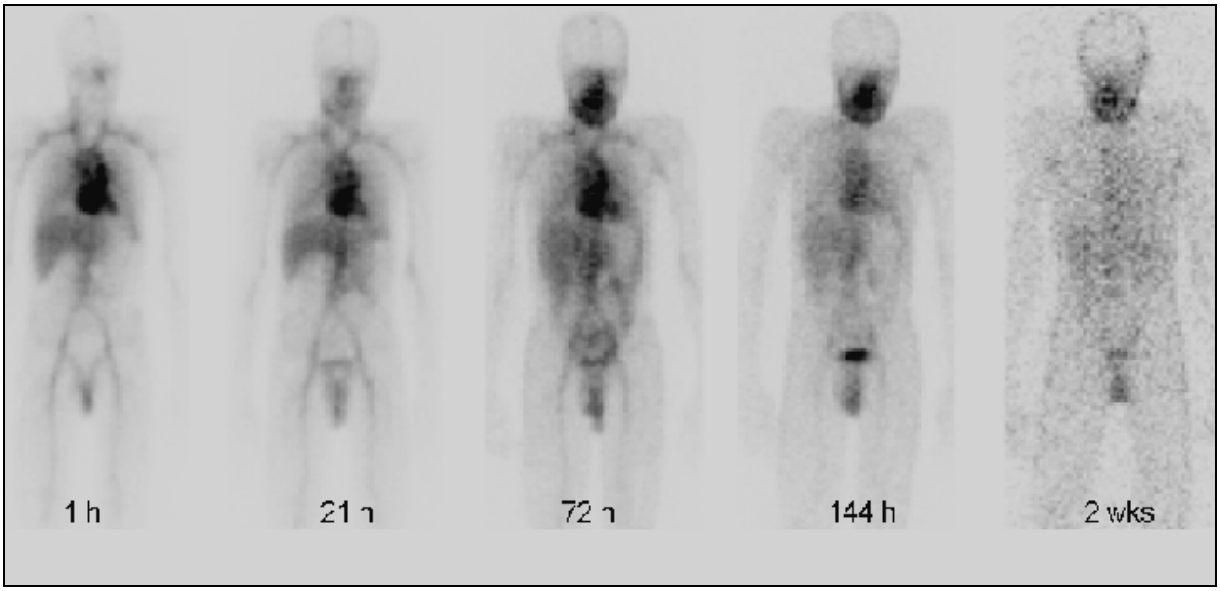

Figure 1. Whole-body scans of patient 4 acquired 1 h after administration of ^{186}Re-cMAb U36, and after 21, 72, and 144 h and 2 wk. Immediately after injection, the most prominent activity is in blood-pool. This activity remains high up to 72 h after injection. Relative uptake of radioimmunoconjugate in tumor in the right oropharynx increases over time. Tumor becomes better delineated as background activity decreases.

Scintigraphy after administration of ^{99m}Tc- and ^{186}Re-cMAb U36 showed a similar biodistribution including comparable uptake at tumor sites in the head and neck (Figure 2). Planar and SPECT imaging at later time intervals with ^{186}Re-cMAb U36 showed improved delineation of small lesions and distant metastases. These concerned mainly lung metastases, of which visualization was hampered by the presence of blood-pool activity, requiring imaging at later intervals (Figure 3). Because of the small size of some lung metastases, not all detected with CT could be detected with planar imaging or SPECT of the chest. Prolonged high blood-pool activity in one patient (patient 13) disturbed the visualization of a tumor in the distal esophagus, because the tumor was directly posterior to the heart. A palpable, cytologically proven, 2x4 cm metastasis in the right axilla of patient 10 was not detected by scintigraphy during RIT. High uptake in the lungs was seen in patient 5 on whole-body scans obtained at 21 h after administration of ^{99m}Tc-cMAb U36. Imaging at 21 h after administration of ^{186}Re-cMAb U36 did not show this high uptake. Patient 7 showed increased uptake in a decubitus lesion on her left shoulder.

Figure 2. Comparison of planar imaging of head and neck region of patient 1 acquired 21 h after administration of ^{99m}Tc-cMAb U36 (left) and ^{186}Re-cMAb U36 (right). Accumulation of radiolabeled cMAb U36 is visible at tumor recurrence in right oropharynx.

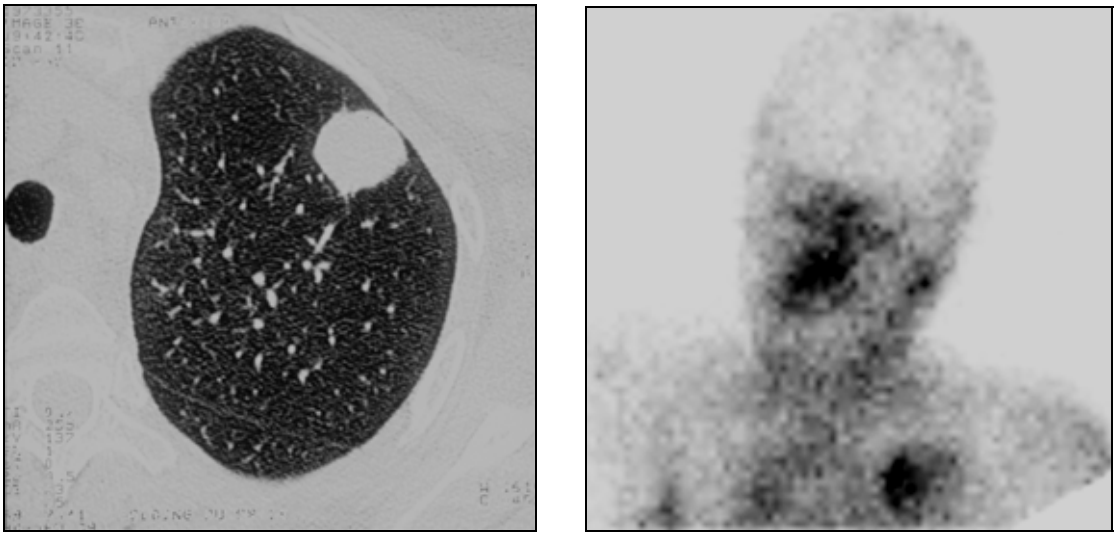

Figure 3. CT scan and planar anterior gamma camera scan of patient 7 obtained 144 h after administration of ^{186}Re-cMAb U36 show targeting of a lung metastasis located in upper lobe of left lung. Accumulation of ^{186}Re-cMAb U36 is also visible on both sides of oropharynx, where recurrent tumor is present.

Pharmacokinetics

HPLC analysis of the conjugates for injection showed a monomeric peak, with more than 97% of the radioactivity bound to cMAb U36. In plasma samples taken after administration of ^{186}Re-cMAb U36, HPLC analysis identified monomeric radiolabeled cMAb U36 (retention time: 15.9 ± 0.2 min) and a second small peak corresponding to less than 5% of the total radioactivity, representing complexed cMAb U36 (retention time: 11.1 ± 1.1 min). Therefore, all radioactivity in plasma was assumed to be monomeric radiolabeled cMAb U36, and pharmacokinetic analysis was based on these data. For ^{186}Re-cMAb U36, in most patients a biexponential whole-blood disappearance could be described with a mean rapid distribution-phase half-life ($t_{1/2}\alpha$) of 7.2 ± 5.1 h, followed by a slower elimination phase half-life ($t_{1/2}\beta$) of 80.0 ± 36.6 h. Accurate assessment of these parameters for ^{99m}Tc-cMAb U36 was hampered by the limited number of measurements at times later than 21 h after injection. The effective AUCs to infinity to be used for bone marrow dosimetry of ^{186}Re-cMAb U36 ranged from 125 to 666 mCi x h/L (4.6 to 24.6 GBq x h/L). Biological AUCs for both ^{99m}Tc- and ^{186}Re-cMAb U36, as determined up to 25 h after injection, showed variability between patients but, if compared for individual patients, showed a strong correlation (r = 0.94, P < 0.01), as is demonstrated in Figure 4.

Human anti-cMAb U36 response

HACA and HAMA immune responses were evaluable for all patients except patient 5. Five of the twelve evaluable patients showed a HACA response (Table 3). In three of these patients elevated HACA levels were observed within 1 wk after the ^{99m}Tc-cMAb U36 study procedure, before the start of ^{186}Re-cMAb U36 RIT. One of these patients (patient 6) already showed detectable HACA levels before cMAb U36 administration. He had never before been exposed to mMAbs or cMAbs in the past. In three patients with an immune response, the highest HACA levels were found 1 wk after the start of RIT. One of the patients with a HACA response also showed elevated HAMA titers, indicating that only in this patient antibodies directed against the murine-variable regions had been formed.

Table 3. Human-anti-cMAb U36 Antibody Response*

		Before RIS		Before RIT		1 wk post-RIT		6 wk post-RIT	
Patient no.	**cMAb U36 dose**	**HAMA titer**	**HACA**	**HAMA titer**	**HACA**	**HAMA titer**	**HACA**	**HAMA titer**	**HACA**
	(mg)		**(mg/L)**		**(mg/L)**		**(mg/L)**		**(mg/L)**
1	2+12	<50	<0.2	<50	1.50	<50	2.05	<50	0.33
2	2+12	<50	<0.2	<50	1.30	113	5.73	ND	ND
3	2+52	<50	<0.2	<50	<0.2	52	<0.2	55	<0.2
4	2+52	ND	ND	<50	<0.2	<50	<0.2	110	<0.2
5	2+52	<50	<0.2	<50	<0.2	ND	ND	ND	ND
6	2+52	<50	0.7	<50	1.21	87	5.29	133	2.46
7	2+52	<50	<0.2	<50	<0.2	<50	<0.2	510	1.76
8	2+52	<50	<0.2	<50	<0.2	<50	<0.2	<50	<0.2
9	2+52	<50	<0.2	67	<0.2	<50	<0.2	119	<0.2
10	2+52	<50	<0.2	<50	<0.2	<50	<0.2	<50	<0.2
11	2+52	<50	<0.2	<50	<0.2	<50	<0.2	147	<0.2
12	2+52	<50	<0.2	<50	<0.2	136	1.88	94	1.32
13	2+52	<50	<0.2	<50	<0.2	<50	<0.2	<50	<0.2

* HAMA titers and HACA levels were measured before administration of 2 mg of ^{99m}Tc-labeled cMAb U36 for RIS and within 1 wk when patients received 12 or 52 mg ^{186}Re-labeled cMAb U36 for RIT. After the start of RIT, HAMA and HACA responses were measured at 1 and 6 wk. Underlined values represent positive responses.

Table 4. Tumor Absorbed Dose Estimates and Response.

Patient no.	Total administered dose	Tumor volume	Total tumor absorbed dose*	Tumor absorbed dose	Response	Follow-up
	(mCi)	(cm3)	(Gy)	(cGy/mCi)		(mo)
1	13	34	2.0	15.7	stable lung metastases	4
2	16	80	1.8	11.4	progression	1
3	19	37	4.1	21.4	progression	4
4	19	81	6.7	34.4	progression	3
5	43	40	3.0	6.9	na	na
6	57	nd	nd	nd	progression	5
7	44	32	nd	nd	stable lung metastases	3
8	46	28	3.5	7.6	stable disease for 6 mo	8
9	46	14	18.1	39.4	progression	4
10	48	74	4.5	9.2	progression	3
11	80	80	16.7	20.8	progression	6
12	59	135	8.3	14.2	reduction of tumor mass	1
13	58	98	nd	nd	reduction of tumor mass	2

Abbrevations: na, not available; nd, not done. * Ten patients were evaluable for tumor dosimetry. The site of origin of the tumors is indicated in Table 1. Only volumes of visualized tumor lesions are included, dosimetry of lung metastases was not possible.

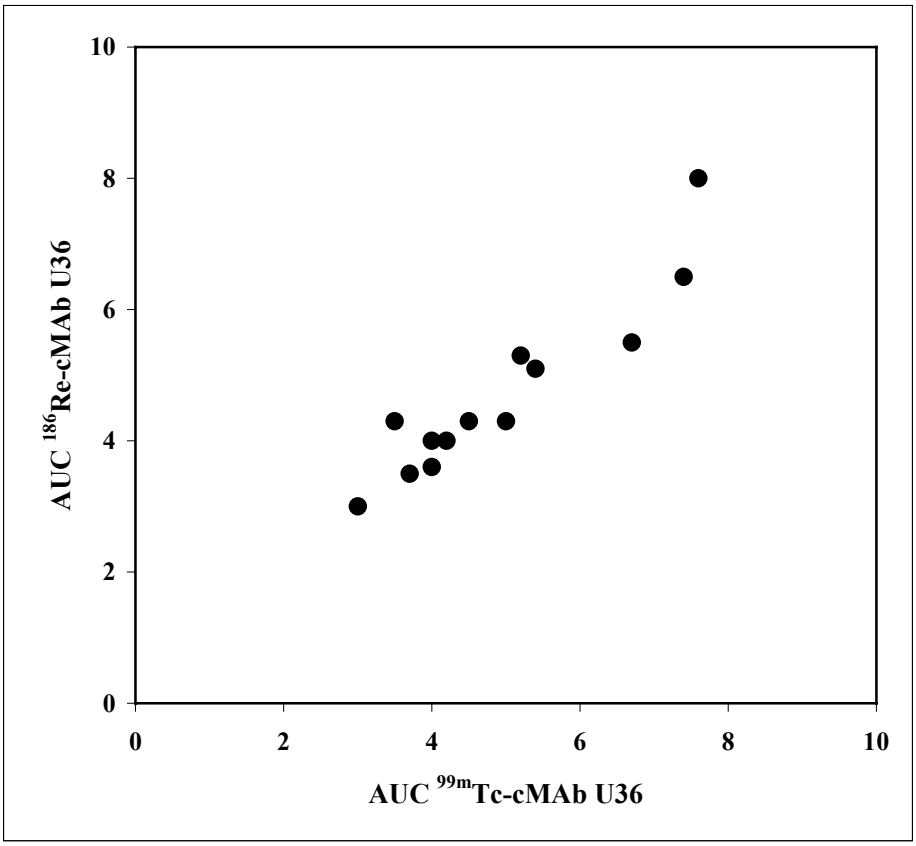

Figure 4. Relationship between the AUC (expressed as %ID x h) determined for 0-25 h after injection for individual patients, both for ^{99m}Tc-cMAb U36 (horizontal axis) and for ^{186}Re-cMAb U36 (vertical axis). Although relatively large variations are seen between patients, pharmacokinetic behaviour of the two conjugates seems similar for individual patients (correlation $r = 0.94$, $P < 0.01$).

Dosimetry

Assuming a homogeneous distribution, absorbed dose to the whole-body could be estimated for twelve patients (Table 2). One patient (patient 7) was not able to complete the series of whole-body images, and planar serial regional imaging was done. The whole-body absorbed self-dose was 1.0 ± 0.2 cGy/mCi (27.0 ± 5.4 cGy/GBq). The mean biological whole-body half-life was 344 ± 164 h. For liver, lungs, spleen, and kidneys, calculated absorbed dose estimates to organs were 4.9, 7.4, 5.1 and 5.0 cGy/mCi (132.4, 200.0, 137.8, and 135.1 cGy/GBq), respectively.

Bone marrow doses, determined from the whole-blood time-^{186}Re activity concentration curve, ranged from 20 to 112 cGy. The mean radiation dose to the bone marrow was 1.9 ± 0.6 cGy/mCi (51.4 ± 16.2 cGy/GBq). The most frequently observed myelotoxicity, that is, the decrease in number of platelets, correlated with

the bone marrow dose ($r = 0.8$ [$P < 0.01$] for platelet nadir and $r = 0.6$ [$P < 0.05$] for the percentage decrease from baseline platelet counts) (Figure 5). A significant correlation was found between the bone marrow dose and the decrease of granulocytes ($r = 0.7$ [$P < 0.05$] for the percentage decrease, and $r = 0.6$ [$P < 0.05$] for the granulocytes nadir). No significant correlation was found for bone marrow dose and decrease in white blood cell count.

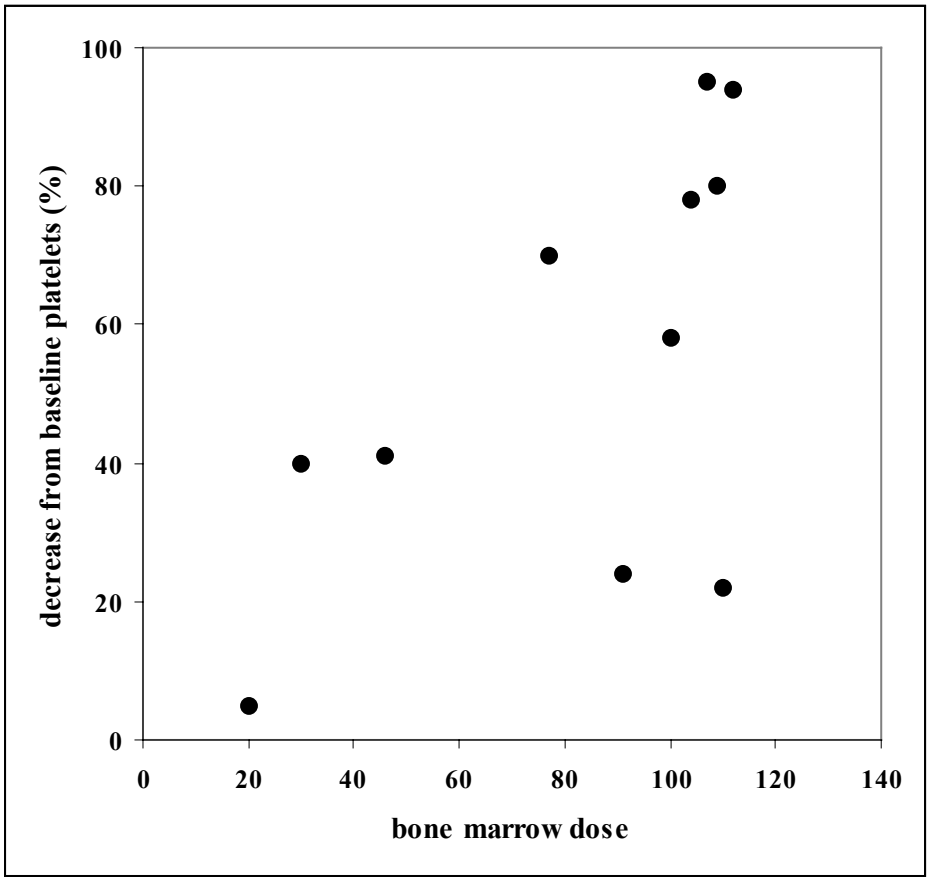

Figure 5. Relationship between bone marrow dose (cGy) derived from whole-blood time-activity concentration curve for ^{186}Re-cMAb U36 and percentage decrease from baseline platelet count (correlation $r = 0.6$, $P < 0.05$).

Tumor dosimetry was possible for ten patients (Table 4). The volumes of these lesions ranged from 14 to 135 cm^3. The ROI method on whole-body scans revealed the highest tumor uptake values at 72 h after injection, with a mean uptake of 18.6 ± 9.1 %ID/kg. The mean tumor absorbed dose was 18.0 ± 11.0 cGy/mCi (486.5 ± 297.3 cGy/GBq), with tumor doses ranging from 3.0 to 18.1 Gy at the MTD. For the ten

patients evaluable for tumor dosimetry, the ratio between tumor absorbed dose and bone marrow absorbed dose was 11.2 ± 8.7.

Tumor response

One patient (patient 8), treated at the dose level of 27 mCi/m^2, had stable disease for 6 mo as assessed with CT. At the highest dose level -41 mCi/m^2-, obvious tumor shrinkage was observed in two patients. A marked reduction in size of a tumor in the esophagus (patient 13) was observed 3 wk after administration of ^{186}Re-cMAb U36 (Figure 6). This tumor partly compressed a stent placed in the esophagus, and after RIT a 60% decrease in tumor size was observed simultaneously with relaxation of the stent. Another patient (patient 11) showed a reduction in size of a bulky tumor recurrence located submentally and of a lymph node metastasis on the right side of the neck. These two antitumor effects were observed in the 41 mCi/m^2 group and did not meet criteria for an objective response because of a short duration. In other patients, who experienced disease progression under previous treatment, RIT stabilized the growth of some of the lesions. These stabilizations did not meet the criteria for objective response and included small lung metastases (patients 1 and 7), that seemed unchanged while other neck lesions progressed.

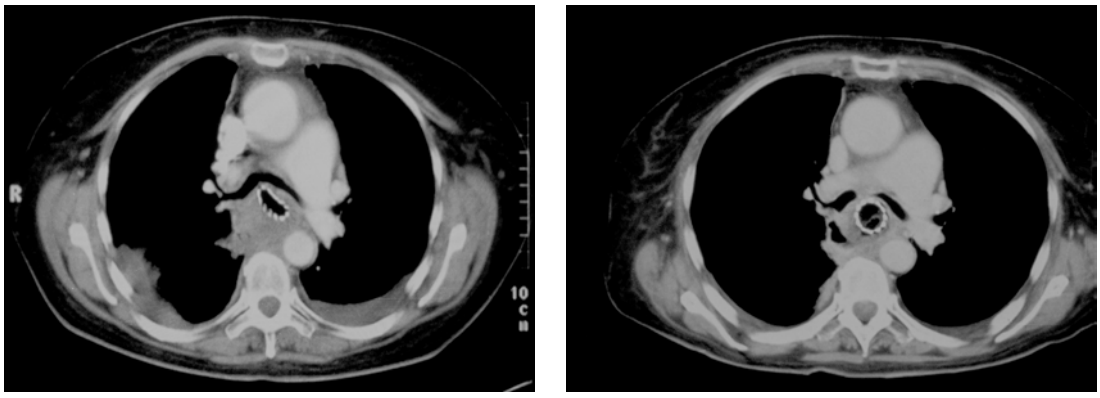

Figure 6. CT scan (left) of patient 13 shows large tumor originating from esophagus compressing stent that was placed for palliation 12 mo before RIT. CT scan (right) of same patient 3 wk after administration of 58 mCi (2.15 GBq) ^{186}Re-labeled cMAb U36. Sixty percent decrease in tumor size was observed as well as relaxation of stent.

Discussion

RIT with ^{186}Re-cMAb U36 seems to be safe, and no side-effects were observed in this phase I study besides dose-limiting myelosuppression at the ^{186}Re dose level of 41 mCi/m^2. The MTD of 27 mCi/m^2 as found for ^{186}Re-cMAb U36 is considerably lower than has been described for other ^{186}Re-labeled MAbs in dose-finding studies. For mMAb NR-LU-10, the MTD was established at 90 mCi/m^2 (3.3 GBq/m^2), while for cMAb NR-LU-13, the MTD was found to be 60 mCi/m^2 (2.2 GBq/m^2) (*11,13*). This difference can be explained by the longer circulating half-life in blood of cMAb U36, compared with the two other radioimmunoconjugates. The mean $t_{1/2}$ß of cMAb U36 was 80.0 h, while for NR-LU-10 and NR-LU-13 a mean $t_{1/2}$ß of 26.5 and 36.5 h, respectively, was found. MAb U36 does not bind to the cellular fraction of the bone marrow, and no patients with bone metastases participated in this study, nor did RIS reveal increased radioactivity uptake in the bone marrow.

Myelotoxicity consisted mainly of thrombocytopenia, with a nadir 4 wk after administration of ^{186}Re-cMAb U36. Grade 4 thrombocytopenia was observed at the highest dose level -41 mCi/m^2- for two patients, whereas grade 3 thrombocytopenia developed in one of five evaluable patients at the MTD level. Leucocytopenia of a grade higher than 3 was seen only at the 41 mCi/m^2 dose level. The development of thrombocytopenia could be related to the bone marrow dose, calculated from the whole blood time-^{186}Re activity curve.

Because cMAb U36 clearance differed considerably between patients in the same dose group and resulted in thrombocytopenia of variable severity, an individualized radiation dose selection for cMAb U36 RIT candidates seems mandatory. In this study, we evaluated the possibility of using ^{99m}Tc-cMAb U36 for prediction of the ^{186}Re-cMAb U36 biodistribution. To limit antigen occupation in the tumor because of such pretherapy studies, ^{99m}Tc-cMAb U36 was administered at a much lower antibody dose than ^{186}Re-cMAb U36: 2 mg versus 52 mg. The overall biodistribution of the conjugates observed with RIS seemed to be the same at 21 h after injection, and comparable targeting of tumor lesions was seen (Figure 2). At later times, imaging with ^{99m}Tc-cMAb U36 is not feasible because of the short half-life time of ^{99m}Tc, which is consistent with the findings of Breitz *et al*., who evaluated the use of ^{99m}Tc-labeled NR-CO-02 (Fab')$_2$ to predict ^{186}Re dosimetry (*29*). Therefore, some distant metastases clearly delineated by ^{186}Re-cMAb U36 RIS at later times could not be visualized reliably by ^{99m}Tc-cMAb U36 RIS. Despite the difference in antibody dose, the pharmacokinetic behavior of the ^{99m}Tc- and ^{186}Re-conjugates appeared to be similar during the first 25 h after injection. The physical half-life time compared to the biological half-life time of ^{99m}Tc-cMAb U36, however,

did not allow accurate prediction at later times. On the basis of these data, we think that prediction of radiation dose delivery to bone marrow, tumor and normal tissues in individual ^{186}Re-cMAb U36 RIT candidates may be feasible, but that because of the short physical half-life of ^{99m}Tc, it is not the ideal radionuclide for use in such a pretherapy procedure. A better candidate may be ^{186}Re itself. Data on radiation dose rates and bone marrow dosimetry from this study indicate that up to 10 mCi (370 MBq) ^{186}Re can be safely used as trace-labeled dose, thus allowing imaging and sampling for pharmacokinetics until 7 days after injection. An advantage of using ^{186}Re is that just one radioimmunoconjugate has to be developed for both pretherapy procedure and RIT.

Besides the level of radiation dose delivery to the bone marrow, other factors may affect the severity of thrombocytopenia on RIT. For example, among the three patients in the 41 mCi/m^2 group, patient 11 received a bone marrow dose similar to that received by patient 12 but showed more severe myelotoxicity (grade 4 thrombocytopenia and granulocytopenia). This result was most likely related to past myelotoxic treatment. In addition, patient 13 suffered from severe myelotoxicity that could be explained neither by a history of myelosuppressive therapy nor by an increased MAb retention. This result indicates that although pretherapy procedures are used for individual bone marrow dosimetry, individual variation in development of myelotoxicity can be considerable.

Previous immunohistochemical studies revealed that high CD44v6 expression in HNSCC tumors is similar to that as in normal squamous epithelia; therefore, we anticipated that acute local toxicity might become apparent in the oral mucosa *(17-19)*. However, despite the fact that most patients previously experienced mucositis after external beam irradiation, none of the patients experienced such toxicity during this RIT study, possibly because the target antigen is less accessible in normal mucosa than in HNSCC. Previous biodistribution studies in which tissue uptake of radiolabeled murine MAb U36 was assessed by biopsy of the surgical specimen revealed a 2.3 times lower uptake of the MAb in normal mucosa than in HNSCC tissue, at 2 and 7 days after injection (*14,15*). Moreover, for dosimetric calculations one must consider that a part of the disintegration energy dissipates outside the distribution volume of the tissue. Maraveyas *et al.* reconstructed a larynx phantom and concluded that, for ^{186}Re, the absorbed fraction in normal mucosal linings will be about 1.6 times less than in HNSCC tissue, leading to a greater tumor *versus* mucosa dose advantage (*34*).

In previous RIS studies with ^{99m}Tc-labeled mMAb U36, HAMA responses were observed in five of nine (56%) patients when using the same criteria for positivity as in this study (*15*). With the aim of reducing immunogenicity, cMAb U36 was constructed. We found HACA responses directed against cMAb U36 in five of

twelve evaluable patients (42%), while in one of these patients (8%) antibodies directed against the murine variable region were found. Although a reduction in the development of HAMA has been achieved (from 56% to 8%) with cMAb U36, our data indicate that in four (maybe five) of these patients antibodies directed against epitopes residing in the human portion or in the murine-human fusion region of the cMAb had developed.

Three patients already showed HACA before the start of RIT. In two of these patients, HACA responses were induced by administration of 2 mg ^{99m}Tc-cMAb U36, one week before the start of RIT, whereas in the third patient an elevated preimmune HACA level was found. In one of these patients (patient 2), a relatively rapid whole-body and blood clearance of ^{186}Re-cMAb U36 was observed, while RIS revealed relatively high accumulation of activity in the liver. This patient had the highest HACA level measured in the study, 5.73 mg/L. The HACA response may be responsible for the rapid blood clearance and liver accumulation of ^{186}Re-cMAb U36 in this patient. The fact that a rapid blood clearance was not observed for the other two patients with increased HACA levels at the start of RIT might have been caused by the lower HACA levels of these patients or the higher cMAb U36 dose (52 mg instead of 12 mg) they received for RIT.

We have not analyzed the patients' sera for human antibodies directed against the MAG3 chelate. However, one can conclude already, at this stage, that immunogenicity of cMAb U36 cannot be neglected when starting new studies with cMAb U36. This conclusion may be particularly true when considering pretherapy studies or repeated dosing.

The observation of antitumor effects in patients with bulky disease offers opportunities for further development of RIT as an adjuvant treatment. Tumor uptake, as assessed by ROIs on scintigraphy, agreed with uptake values obtained previously with mMAb U36 (*14,15*). The calculated tumor absorbed doses ranged from 3.0 to 18.0 Gy for the 27 mCi/m^2 dose group, whereas the mean absorbed dose to the tumor was 18.0 cGy/mCi (486.5 cGy/GBq) for all evaluable patients. As shown in Table 4, these tumor doses appear sufficient for antitumor effects. That antitumor effects are caused by immune modulating effects is unlikely, because cMAb U36 was shown to lack antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)-mediating activity *in vitro* (*15*). RIT studies with ^{186}Re-mMAb E48 in HNSCC-bearing nude mice revealed that with an accumulated dose in the range of 11 - 34 Gy, complete remissions of 18 - 50% could be achieved (*35*). Breitz *et al.* found a partial response lasting 7 mo on treatment of a colon cancer patient with two cycles of ^{186}Re-labeled NR-LU-10. Tumor doses were 21 and 7 Gy for the two cycles (*11*). These data indicate that RIT with ^{186}Re-cMAb U36 in its current form occasionally can cause antitumor effects. However, for complete tumor eradication

the radiation delivery to the lesions should be increased several times, as when applying RIT in an adjuvant setting for treatment of minimal residual disease. Recently, we reported on the relationship between HNSCC size and MAb accumulation (*36*). Data for this report were obtained from several RIS and biodistribution studies with the anti-HNSCC MAbs E48 and U36 in HNSCC patients. MAb uptake in small-volume tumors (1 cm^3) was found to be approximately four times higher than uptake in large-volume tumors (> 50 cm^3). No data are available for MAb uptake in tumor deposits smaller than 1 cm^3, but we nevertheless think that the data presented in this article justify the evaluation of RIT with ^{186}Re-cMAb U36 in an adjuvant setting.

Several other strategies are followed at our institute to improve the efficacy of RIT. Most appealing may be strategies with higher doses of ^{186}Re-cMAb U36 combined with peripheral stem cell re-infusion or a combination of RIT with inhibitors of the epidermal growth factor receptor (EGFR). In the latter approach, RIT is combined with MAbs or chemical inhibitors capable to block EGFR. In a recent study, we showed that the anti-EGFR MAb 425 strongly enhanced the efficacy of RIT with ^{186}Re-cMAb U36 in HNSCC-bearing nude mice (*37*). Taking into account that high expression of EFGR in most HNSCCs and its absence on bone marrow cells, this approach holds promise for future clinical application.

Conclusion

RIT with ^{186}Re-cMAb U36 seems safe. The pharmacokinetics of ^{186}Re-cMAb U36 can be predicted by ^{99m}Tc-cMAb U36, thus creating the possibility of such a procedure for selection of a safe RIT dose. However, such pretherapy procedure may induce a HACA reponse. This study shows that tumoricidal doses of ^{186}Re-cMAb U36 can be reached in HNSCC patients with bulky disease. Avenues for RIT are thus opened, especially when applied in an adjuvant setting.

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3

Evaluation of limited blood sampling in a preceding ^{99m}Tc-labeled diagnostic study to predict the pharmacokinetics and myelotoxicity of ^{186}Re-cMAb U36 radioimmunotherapy.

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Abstract

^{186}Re-labeled chimeric monoclonal antibody U36 (cMAb U36) was recently evaluated in a phase I dose escalation study in head and neck cancer patients. All thirteen patients received ^{99m}Tc-cMAb U36 before ^{186}Re-cMAb U36 radioimmunotherapy. The aim of this study was to evaluate the suitability of multiple or limited blood sampling to predict clearance, bone marrow absorbed dose, and myelotoxicity of ^{186}Re-cMAb U36.

Population pharmacokinetics of ^{186}Re-cMAb U36 were analysed with a nonparametric expectation algorithm (NPEM 2) and used for Bayesian analysis of individual patient data to predict cMAb U36 clearance.
^{186}Re-cMAb U36 clearance was most accurately predicted (r = 0.91, P < 0.001) with limited sampling for sample points 4 and 72 h after administration of ^{186}Re-cMAb U36. These predictions were less accurate with ^{99m}Tc-cMAb U36 (r = 0.51, P = 0.078, for multiple sampling; r = 0.47, P = 0.104 for sampling at 4 and 21 h after administration). Thrombocytopenia was found to be correlated with the bone marrow absorbed dose and was equally well predicted by limited blood sampling after administration of ^{99m}Tc-cMAb U36 (r = 0.81, P < 0.01) or ^{186}Re-cMAb U36 (r = 0.79, P < 0.01).

In conclusion, limited sampling seems useful to predict pharmacokinetics and myelotoxicity of ^{186}Re-cMAb U36.

Introduction

Radiogenic damage to the bone marrow is the dose limiting toxicity in most of the radioimmunotherapy (RIT) studies conducted thus far, resulting in thrombocytopenia and leucocytopenia with a nadir at 4-6 wk after therapy. The bone marrow absorbed dose is often found to be related to the severeness of myelotoxicity (*1*). In a previous paper (*2*) we report on a phase I RIT trial in which ^{99m}Tc-labeled chimeric MAb U36 (cMAb U36) was administered one week before ^{186}Re-cMAb U36 to head and neck cancer patients. With these radioimmunoconjugates, an intrapatient consistency of pharmacokinetics was demonstrated for the decay-corrected areas under the curve (AUCs) up to 25 h after administration. Due to the short half-life of ^{99m}Tc in comparison to ^{186}Re, an accurate estimation of the total AUC based on individual patient data was not possible. In this study, a population-based model was used to compare the data on ^{99m}Tc-cMAb U36 and ^{186}Re-cMAb U36 pharmacokinetics obtained from the same group of patients. Subsequently, the potential of limited blood sampling to predict the pharmacokinetics of ^{186}Re-cMAb U36 and the severity of myelotoxicity after RIT, was evaluated.

Materials and methods

Patients and study design

The study was approved by the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands), and informed consent was obtained. Study design and methods including antibody production, radiolabeling, safety, imaging, and efficacy data of the trial have been reported previously *(2)*. Prior to RIT, patients received 740 MBq ^{99m}Tc-labeled cMAb U36 (2 mg), followed one week later by a single dose of ^{186}Re-cMAb U36 (12 or 52 mg) in radiation dose escalating steps of 0.4, 1.0, and 1.5 GBq/m^2. Blood samples were collected at 5, 10, and 30 min, and 1, 2, 4, 16, and 21 h after injection in both studies, as well as at 72 h in the RIT study. Hematologic parameters were obtained on a weekly basis for at least 6 wk, or until recovery of myelotoxicity was observed.

Pharmacokinetic modeling, bone marrow dosimetry, and myelotoxicity

Blood samples were counted in a multiwell gamma counter (1470 Wizard, Wallac, Turku, Finland), and compared to an aliquot retained from the conjugate preparation for injection. These results, together with patient age, sex, weight, body surface area, and serum creatinine, were analysed with a nonparametric expectation maximization algorithm (NPEM 2, USC-Pack, Los Angeles, CA) to reveal the

following mean pharmacokinetic parameters: initial volume of distribution (V_d), elimination constant rate (k_{elm}), and transfer rate constants (k_{12} and k_{21}) (*3*). The individual pharmacokinetic parameters were calculated with MW/Pharm (Mediware, Groningen, The Netherlands) using the population pharmacokinetic parameters as Bayesian forecast. Subsequently, the following analyses were performed:

1. Bayesian clearance estimates of cMAb U36 were assessed, using blood activity concentration data of either ^{99m}Tc-cMAb U36 or ^{186}Re-cMAb U36. For these estimates data of either all blood samples ("multiple sampling") or combinations of two blood samples ("limited blood sampling") were used.
2. The obtained Bayesian clearance estimates were used to calculate the effective clearance according to the following equation: $Cl_{effective} = Cl_{biological} + Cl_{phys}$, where $Cl_{effective}$ and $Cl_{biological}$ are the effective and biological clearance of the radioimmunoconjugate, respectively, and Cl_{phys} is the physical clearance of the radionuclide (V_d x ln 2 / [half-life of ^{186}Re] ; half-life of ^{186}Re = 90.62 h.
3. The actual effective clearance (i.e. non-Bayesian clearance) was assessed by fitting blood activity concentration data of all blood samples of an individual patient obtained after administration of ^{186}Re-cMAb U36.
4. Bayesian clearance estimates were compared with the actual non-Bayesian clearance.

To calculate the total AUC in blood (AUC_{blood}), and subsequently the bone marrow absorbed dose, the following equation was used:

$$AUC_{BLOOD} = ID / CL_{EFFECTIVE},$$

where ID is the total injected therapy dose. This AUC_{blood}, together with the total body radioactivity, was used for calculation of a patient-specific bone marrow dose according to the method of Shen *et al*. (*4*). The bone marrow absorbed dose estimates were compared with the development of myelotoxicity, that is, the nadir and the percentage decrease from baseline values of platelets, white blood cell count, and granulocytes.

Statistical analysis

All mean values reported represent arithmetic means with corresponding SDs. Associations between variables were calculated with SPSS 7.5 software (SPSS Inc., Chicago, IL) using Pearson correlation tests with *P*-values < 0.05 considered statistically significant. Multiple regression analysis was performed to find a possible

relationship between the covariates age, weight, body surface area, serum creatinine, injected dose, clearance, and bone marrow absorbed dose in predicting myelotoxicity as determined by the nadir of platelets.

Results

Patient characteristics are shown in Table 1. The mean population parameters obtained with NPEM 2 were k_{elm} : 0.013 h^{-1} (± 0.006), k_{12} : 0.024 h^{-1} (± 0.018), k_{21} : 0.215 h^{-1} (± 0.146), and V_d : 0.074 L/kg (± 0.011). A mean cMAb U36 clearance of 0.056 L/h (range 0.036 - 0.074 L/h) was found in the therapy study with ^{186}Re-cMAb U36 using non-Bayesian analysis. The goodness of fit of the Bayesian and non-Bayesian ^{186}Re-cMAb U36 clearance was assessed (r = 0.99, P < 0.0001).

Table 1. Patient Characteristics

Patient no.	Age/Sex	Weight (kg)	BSA (m^2)	Prior chemotherapy	Injected Dose ^{186}Re-cMAb U36 (mCi)	BM dose (cGy)
1	53/M	55	1.63	Yes	13	46
2	54/F	48	1.50	None	16	25
3	54/M	67	1.78	None	19	30
4	47/M	57	1.78	None	19	20
5	56/M	58	1.63	Yes	43	73
6	66/M	86	2.03	Yes	57	109
7	56/F	57	1.59	None	44	91
8	58/M	61	1.76	None	46	77
9	67/M	63	1.75	None	46	110
10	68/M	69	1.86	None	48	100
11	52/M	82	2.01	None	80	104
12	52/M	47	1.46	Yes	59	107
13	59/F	58	1.56	None	58	112

Abbreviations: M, male; F, female; BSA, body surface area; BM, bone marrow.

Bayesian analysis using data from all sample points in the ^{99m}Tc-cMAb U36 study was not able to predict with statistical significance the actual ^{186}Re-cMAb U36 clearance for an individual patient (r = 0.51, P = 0.078). The optimal ^{99m}Tc-cMAb U36 sample pair for prediction was 4 and 21 h after administration (r = 0.47, P = 0.104).

The combination of sample points at 4 and 72 h after administration of ^{186}Re-cMAb U36 appeared to be the optimal sample pair for prediction of the actual clearance of ^{186}Re-cMAb U36 ($r = 0.91$, $P < 0.001$).

Development of hematologic toxicity was evaluable for eleven out of thirteen patients, since two patients did not complete the follow-up for evaluation of myelotoxicity. Correlations between myelotoxicity and bone marrow absorbed dose estimates are given in Table 2. The most severe toxicity, that is, thrombocytopenia, was shown to be correlated with the bone marrow dose calculated from the actual ^{186}Re-cMAb U36 clearance: $r = 0.80$, $P < 0.01$, and $r = 0.66$, $P < 0.05$, for platelet nadir and percentage decrease, respectively. If the bone marrow dose was calculated from the Bayesian estimate of ^{186}Re-cMAb U36 clearance obtained from the ^{99m}Tc-cMAb U36 study, and normalized with respect to the injected dose of ^{186}Re-cMAb U36 RIT, these correlations appeared to be similar. The relationship between the predicted bone marrow doses and development of thrombocytopenia is illustrated in Figure 1.

Multiple regression analysis did not show any significant influence of the covariates age, weight, body surface area, or serum creatinine on ^{186}Re-cMAb U36 clearance. The Pearson correlation coefficients for platelets nadir and the different bone marrow dose estimates using multiple and limited sampling were all higher than for total injected dose (Table 2). The individual bone marrow dose estimate was thus a better predictor of myelotoxicity than total injected dose.

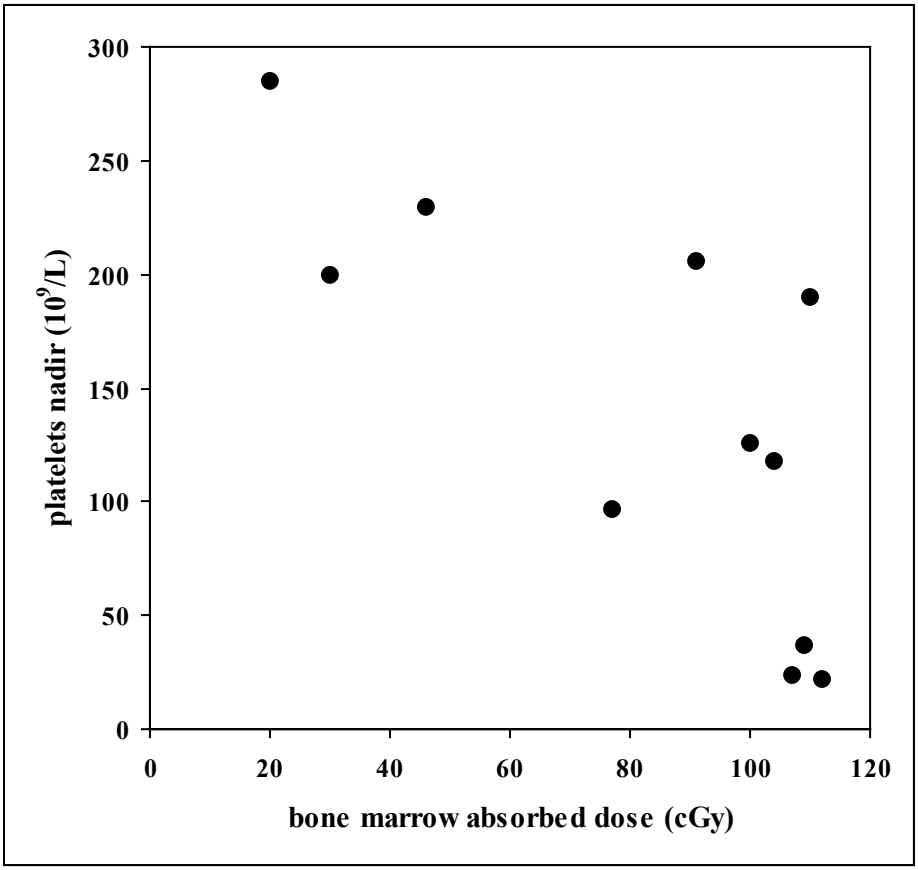

Figure 1. Relationship between bone marrow absorbed dose estimates and development of thrombocytopenia after ^{186}Re-cMAb U36 radioimmunotherapy. Bone marrow absorbed dose estimate was calculated using Bayesian analysis of limited blood sampling at 4 and 72 h after administration of ^{186}Re-cMAb U36.

Table 2. Pearson Correlation Tests for the Prediction of Myelotoxicity.

	Platelet nadir		Platelet decrease (%)		White Blood Cell Nadir		White Blood Cell Decrease (%)		Granulocytes Nadir		Granulocytes Decrease (%)	
	r	*P*	*r*	**P**	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
^{186}Re-cMAb U36, non-Bayesian	0.80	< 0.01	0.66	< 0.05	0.35	0.30	0.49	0.13	0.60	< 0.05	0.68	< 0.05
^{186}Re-cMAb U36, Bayesian, 2 samples	0.79	< 0.01	0.65	< 0.05	0.36	0.27	0.51	0.11	0.61	< 0.05	0.67	< 0.05
^{99m}Tc-cMAb U36, Bayesian, all samples	0.81	< 0.01	0.68	< 0.05	0.44	0.18	0.59	0.06	0.58	0.06	0.68	< 0.05
^{99m}Tc-cMAb U36, Bayesian, 2 samples	0.81	< 0.01	0.67	< 0.05	0.45	0.16	0.55	0.08	0.57	0.07	0.61	< 0.05

Pearson correlation tests to measure correlation between bone marrow absorbed dose estimates and myelotoxicity. Bone marrow absorbed dose was calculated using all blood samples (multiple sampling) and two blood samples (limited sampling) obtained after administration of ^{99m}Tc- and ^{186}Re-cMAb U36.

Discussion

The bone marrow absorbed dose, determined from the blood time-activity curve, is often a good predictor of myelotoxicity (*1*). For patient-specific dose planning, accurate bone marrow absorbed dose estimation might require frequent sampling, which is a burden for patients, especially when performed in an outpatient setting. In the present study, prediction of ^{186}Re-cMAb U36 clearance proved to be feasible when only two instead of all blood samples were selected for Bayesian analysis. This prediction was optimal for the sample pair at 4 and 72 h after administration of ^{186}Re-cMAb U36. All patients underwent a preceding pretherapy study with ^{99m}Tc-cMAb U36, but unfortunately, the prediction of ^{186}Re-cMAb U36 clearance from this pretherapy study using Bayesian analysis of multiple or limited blood samples was less accurate.

Pharmacokinetics of ^{99m}Tc-cMAb U36 and ^{186}Re-cMAb U36 could be different as a result of development of human-anti-chimeric antibody (HACA) responses, which were found in five of thirteen patients (*2*), of which three had an onset in the week between the ^{99m}Tc- and ^{186}Re-cMAb U36 administrations. Because HACA titers were low (< 1.50 mg/L) and the amount of administered ^{186}Re-cMAb U36 relatively high (50 mg), the effect of HACA on^{186}Re-cMAb U36 pharmacokinetics, however, is expected to be small. Presence of antigen at nontumor sites and different antibody doses used for pretherapy and therapy studies (as was the case in the present study) can also explain variation in pharmacokinetics. Previous biodistribution studies with MAb U36, however, showed consistency of pharmacokinetics irrespective of the antibody was administered at a dose of 2, 12 or 52 mg (*5*).

Another explanation for the different pharmacokinetics might be that the short half-life of ^{99m}Tc makes the pretherapy procedure with ^{99m}Tc-cMAb U36 of limited value. The short half-life of ^{99m}Tc does not allow sampling later than approximately 25 h after administration. Therefore, ^{99m}Tc-cMAb U36 might allow accurate prediction of the early part of the ^{186}Re-cMAb U36 clearance, but not of the late part. Indeed, biological AUCs for ^{99m}Tc- and ^{186}Re-cMAb U36, as determined up to just 25 h after injection, showed a very strong correlation ($r = 0.94$; $P < 0.01$), confirming intrapatient consistency of pharmacokinetics.

Taking the aforementioned information into account we hypothesized that ^{186}Re-cMAb U36 might be a better candidate for such a pretherapy procedure. ^{186}Re allows sampling at later time points, resulting in a more reliable prediction of cMAb U36 clearance, which was confirmed in this study: ^{186}Re-cMAb U36 clearance was accurately predicted ($r = 0.91$, $P < 0.001$) with limited sampling at 4 and 72 h after administration. However, these sampling points were selected from the therapy data,

and not from a preceding diagnostic study. Whether the same accuracy can be found if the pretherapy study is performed with ^{186}Re-cMAb U36, has to be shown.

Conclusion

This study shows that limited sampling during a pretherapy procedure is a realistic option to predict the ^{186}Re-cMAb U36 clearance during RIT. Development of myelotoxicity after ^{186}Re-cMAb U36 RIT correlated well with the derived bone marrow dose estimates. ^{186}Re-cMAb U36 seems to be better qualified for such a pretherapy study procedure than ^{99m}Tc-cMAb U36, because of its matched half-life.

Acknowledgements

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4

Re-infusion of unprocessed, granulocyte colony-stimulating factor (G-CSF)-stimulated whole blood allows dose escalation of ^{186}Re-labeled chimeric monoclonal antibody U36 radioimmunotherapy in a phase I dose escalation study

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Abstract

In an earlier phase I radioimmunotherapy (RIT) study with ^{186}Re-labeled chimeric MAb U36 (cMAb U36) in patients with refractory head and neck squamous cell carcinoma (HNSCC), the maximum tolerated dose (MTD) was established at 27 mCi/m^2 (1.0 GBq/m^2), at which bone marrow doses ranged from 0.7 to 1.1 Gy. In the present study, further dose escalation in RIT was evaluated using a facile method of re-infusion of granulocyte colony-stimulating factor (G-CSF) stimulated unprocessed whole blood.

Nine patients with recurrent or metastatic HNSCC were treated at radiation dose levels of 27, 41, and 54 mCi/m^2 (1.0, 1.5, and 2.0 GBq/m^2). Before RIT, G-CSF (10 μg/kg/day) was administered subcutaneously at home during 5 days. On day 6, just prior to administration of ^{186}Re-cMAb U36, 1 L of whole blood was harvested and kept unprocessed at 4° C, until re-infusion after 72 h. Blood samples were taken for analysis of pharmacokinetics and bone marrow dosimetry. Patients were evaluated for myelotoxicity and tumor response.

Blood harvesting, RIT, and re-infusion of whole blood were well tolerated by all patients. G-CSF stimulation resulted in a mean of 0.41 x 10^6 CD34$^+$ cells/kg (range: 0.15-0.83 x 10^6 cells/kg) and a mean committed colony-forming units granulocyte-macrophage (CFU-GM) count of 5.62 x 10^4/kg (range: 0.62-13.37 x 10^4/kg). The mean biological half-life of ^{186}Re-cMAb U36 in blood was 72.6 ± 16.0 h, and bone marrow doses ranged from 2.1 – 2.8 Gy at the highest dose level. Myelotoxicity exceeding grade 3 was not observed. Stable disease was observed in five of nine patients, ranging from 3 to 5 mo, and was still ongoing in one of these patients.

In conclusion, this study indicates that a doubling of the MTD and bone marrow absorbed dose of ^{186}Re-cMAb U36 can be achieved using re-infusion of G-CSF-stimulated unprocessed whole blood.

Introduction

Radiolabeled monoclonal antibodies (MAbs) can be used for selective delivery of radiation to tumor sites. For selective treatment of head and neck squamous cell carcinoma (HNSCC), radioimmunotherapy (RIT) might be an interesting approach as an adjuvant therapy, because there is still a high failure rate, either locally or at distant sites, after locoregional treatment of HNSCC with surgery and/or radiotherapy. In patients with hematological malignancies RIT has shown efficacy, resulting in improved remission and response rates (*1,2*). In most of the RIT trials conducted thus far, radiogenic damage to the bone marrow has been dose limiting, resulting in thrombocytopenia and granulocytopenia occurring with a nadir of 4 to 6 wk after RIT. Autologous transplantation of bone marrow or separated growth factor-mobilized blood stem cells can reduce myelotoxicity and allowed dose escalation of RIT (*3-5*). Stem cell transplantation in patients with relapsed B-cell lymphomas treated with high-dose RIT allowed bone marrow absorbed doses as high as 6.4 Gy, before cardiopulmonary dose-limiting toxicity was observed (*6*).

Both transplantation of bone marrow and filtered blood stem cells require equipment and laboratory facilities for separation and cryopreservation, while both techniques are laborious and expensive. In comparison, granulocyte colony-stimulating factor (G-CSF) stimulated unprocessed whole blood might be an alternative source of blood stem cells. Re-infusion of G-CSF-stimulated whole blood is a straightforward and safe procedure, and can be performed in a routine clinical setting at low cost (*7,8*). The G-CSF stimulated whole blood can be stored for at least 72 h at 4° C, without significant loss of viability of the blood stem cells (*9,10*). While up to 90% of the $CD34^+$ population shows early apoptotic changes after cryopreservation (*11*), only 5-10% apoptotic changes were found in CD34+ population in whole blood transplants after 72 h storage (*12*). In multicyclic chemotherapy regimens of short duration this procedure allowed dose intensification in patients with small-cell lung cancer (*7,13*). Moreover, myeloablative chemotherapy with whole blood rescue for patients with high-risk lymphoma and multiple myeloma was proven to be feasible (*8,14,15*). To our knowledge, this whole blood procedure has never been applied in RIT studies.

Because the time interval between RIT and the development of myelotoxicity is 4-6 wk, re-infusion of G-CSF stimulated whole blood is a realistic option to reduce myelotoxicity of RIT, because it allows the re-infused blood stem cells to home and proliferate in the bone marrow before myelotoxicity becomes manifest. A prerequisite for this approach is that most of the radioactivity has disappeared from blood and bone marrow at the time of re-infusion. In an earlier phase I study, ^{186}Re-cMAb U36

was evaluated in patients with refractory HNSCC (*16*). The maximum tolerated dose (MTD, at which a grade 4 hematological or grade 3 non-hematological toxicity developed in not more than one of six patients) was found to be 27 mCi/m^2 (1.0 GBq/m^2), with a maximum tolerated bone marrow dose of 0.9 ± 0.2 Gy. Dose limiting grade 4 myelotoxicity occurred in two of three patients treated with 41 mCi/m^2 (1.5 GBq/m^2). The effective half-life of ^{186}Re-cMAb U36 in blood was 37 h, meaning that after 72 h, 25% of the injected radioactivity was still present in the blood. This effective half-life is related to both the biological half-life of the MAb and the physical half-life of the radionuclide, according to the equation [$1/T_{1/2}$ effective = $1/T_{1/2}$ biological + $1/T_{1/2}$ physical]. In the current study, we evaluated whether re-infusion of G-CSF stimulated unprocessed whole blood after 72 h allows dose escalation of ^{186}Re-cMAb U36 RIT.

Materials and methods

Nine patients (five men and four women; age range, 48-71 y) were included in this study. Their characteristics are listed in Table 1. All patients had clinical evidence of relapsed HNSCC, either locoregionally or at distant sites, for which no curative options were available. A histologically confirmed HNSCC in the past was required for inclusion. Other eligibility criteria were described in an earlier report on a phase I dose escalation study without blood stem cell support (*16*). The study was approved by the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands). All patients gave written informed consent after thorough explanation of the study.

The antigen recognized by MAb U36 (Centocor B.V., Leiden, The Netherlands) is the keratinocyte-specific CD44 splice variant epican. The epitope is located in the v6 domain of CD44 (*17*). CD44v6 is expressed by squamous cell carcinomas of the head and neck, lung, skin, esophagus, and cervix, and also by adenocarcinomas of the breast, colon, lung, and stomach (*18,19*). Antibody production, radiolabeling, and quality controls have been described in detail (*16*).The radiochemical purity of ^{186}Re-cMAb U36 ranged from 95 to 97 % as assessed by thin layer chromatography (TLC) of the final product. The immunoreactive fraction of ^{186}Re-cMAb U36 ranged from 89.0 – 93.8 %.

To mobilize blood stem cells, G-CSF (Filgrastim, Neupogen®, Amgen, Thousand Oaks, CA) at a dosage of 10 μg/kg for 5 days was administered subcutaneously at home. On day 6, just prior to RIT, two phlebotomies of 500 mL each were performed via an antecubital vein within 2-3 h. Patients were carefully monitored for blood pressure and heart rate during the procedure, and infusion of 500

mL 0.65 % saline was performed after each phlebotomy. Blood was collected in two 2,3-ethyl, hexyl-phtalate plasticized transfer bags (Nederlands Productie Laboratorium voor Bloedtransfusieapparatuur en Infusievloeistoffen BV, Emmer-Compascuum, The Netherlands) containing 70 mL of CPD as anticoagulant. After collection the bags were sealed, and stored unprocessed and unshaken at 4°C in a temperature-controlled refrigerator. Re-infusion of the unprocessed whole blood was performed after 72 h. The number of cells expressing the CD34 antigen and the committed colony-forming units granulocyte-macrophage (CFU-GM) in stored whole blood were assessed from a sample taken just prior to re-infusion (*10*).

Just after collection of G-CSF-stimulated whole blood 50 mg of ^{186}Re-cMAb U36 was administered intravenously in 5 minutes. Blood samples were collected from the opposite antecubital vein and counted in a multiwell gamma counter (1470 Wizard; Wallac, Turku, Finland) for pharmacokinetic analyses and patient-specific bone marrow dosimetry according to Shen *et al.* (*20*). This method takes the contribution of other organs and the whole-body into account. The dose levels were 1.0, 1.5, and 2.0 GBq/m^2 body surface area, and 3 patients were treated at each dose level. Vital functions (blood pressure, pulse rate, breathing rate, and temperature) were assessed before administration, and after 20, 40, 60, 120, and 240 min. Patients were admitted for 21 h in a special treatment room at the department of nuclear medicine. Thereafter, they stayed in a single room at the otolaryngology/head and neck surgery ward until re-infusion. Scintigraphic imaging studies were performed with a large field-of-view gamma camera (Dual Head Genesys Imaging System; ADAC Laboratories, Milpitas, CA). Whole-body, and lateral, anterior, and posterior planar images and SPECT of the head and neck region were obtained in all patients. Patients were discharged after the re-infusion was completed.

Hematological parameters were obtained at least weekly until recovery of myelotoxicity was observed. The severity of toxicity was graded according to the National Cancer Institute Common Toxicity Criteria (*21*). Data on bone marrow dosimetry and development of myelotoxicity were compared to those from patients treated in an earlier study of ^{186}Re-cMAb U36 without the use of whole blood re-infusion (*16*). Physical examination and CT or MR imaging were performed every 4 wk for evaluation of response. Stable disease was defined as a 50% reduction or a 25% or smaller increase in the sum of the perpendicular diameter products and no new lesions, which had to persist for at least 3 mo. Progression was defined as an unequivocal increase in size (>25%) of any lesion or the appearance of a new lesion.

All mean values reported represent ranges, arithmetic means, and corresponding SD.

Table 1. Patient characteristics.

Patient no.	Age/ Sex	Disease	Prior Treatment	WBC count	$CD34^+$ cells	CFU-GM
	(y)			(x 10^9/L)	(x 10^6/kg)	(x 10^4/kg)
1	60/F	neck recurrence, lung, and liver metastases of laryngeal carcinoma	SURG/XRT	35.6	0.28	6.33
2	48/M	bone metastases of pharyngeal carcinoma	SURG/XRT	44.4	0.17	0.62
3	58/M	local recurrence, lung metastases of piriform sinus carcinoma	CHEM/XRT	44.5	0.30	1.52
4	58/M	neck recurrence, lung, and liver metastases of supraglottic laryngeal carcinoma	SURG/XRT	36.6	0.15	3.28
5	54/F	neck recurrence, lung metastases of tonsillar carcinoma	SURG/XRT	26.6	0.26	6.74
6	57/M	neck recurrence, lung metastases after neck lymph node metastases of unknown primary tumor	SURG/XRT	71.6	0.68	2.35
7	71/F	cutaneous metastases after neck lymph node metastases of unknown primary tumor	SURG/XRT	48.8	0.66	13.37
8	67/F	local recurrence of tonsillar carcinoma	XRT	38.4	0.38	5.34
9	64/M	lung metastases of piriform sinus carcinoma	XRT/SURG	59.2	0.83	10.99

Abbreviations: SURG, surgery; XRT, radiotherapy; CHEM, chemotherapy (cisplatin and 5-FU); WBC, white blood cell; CFU-GM, colony-forming units granulocyte macrophage. WBC count was assessed just prior to RIT, $CD34^+$ and CFU-GM were assessed in stored whole blood just prior to re-infusion.

Results

G-CSF treatment for blood stem cell mobilization did not cause side-effects in any of the patients. The mean white blood cell count after G-CSF stimulation was 45.1 x 10^9/L (range: 26.6 – 71.6 x 10^9/L) (Table 1). Whole blood harvesting did not lead to significant hypotension in patients, and administration of ^{186}Re-cMAb U36, as well as re-infusion of whole blood, was well tolerated by all patients, without occurence of acute side-effects. Scintigraphy showed selective targeting of tumor lesions in all patients, and no accumulation at nontumor sites, except for presence of radioactivity in feces and urine (Figure 1). The mean number of $CD34^+$ cells harvested after G-CSF stimulation was 0.41 x 10^6 cells/kg body weight (range: 0.15-0.83 x 10^6 cells/kg), and a mean CFU-GM count of 5.62 x 10^4/kg was found (range: 0.62-13.37 x 10^4/kg) (Table 1). Pharmacokinetic analysis revealed a mean biological half-life of 72.6 ± 16.0 h for ^{186}Re-cMAb U36 in blood. Bone marrow doses ranged from 0.8 to 2.8 Gy, with a mean bone marrow dose of 0.64 ± 0.13 Gy/GBq (Table 2).

The nadir of platelets was observed 4-5 wk after administration of ^{186}Re-cMAb U36, and the nadir of white blood cells and granulocytes was observed after 5-6 wk. In none of the patients a platelet count of less than 20 x 10^9/L was observed. For three patients, platelets were below 50 x 10^9/L for 5 to 21 days (Table 2). The granulocyte nadir was below 1.0 x 10^9/L in four patients lasting for 2, 4, 7, and 11 days, respectively. Three patients required transfusion of packed red blood cells for low hemoglobin levels, which was regarded as grade 3 toxicity. In one of these patients (patient 1) the transfusion was performed because of low hemoglobin level shortly after re-infusion of the unprocessed whole blood, which was therefore most likely the result of hemolysis rather than radiation-induced. Patient 4 was given 3 packed red blood cells as well as a single transfusion of platelets, because of a sudden low hemoglobin level (4.5 mmol/L) at 4 wk after RIT, which was attributed to a gastro-intestinal bleeding, caused by concomitant use of corticosteroids and nonsteroidal anti-inflammatory drugs. At the time of the transfusion there was a grade 3 thrombocytopenia, which showed recovery within 5 days. The other patient (patient 6) was given two transfusions of 3 packed red blood cells for hemoglobin levels of 5.2 and 5.5 mmol/L at 3.5 and 4 wk after RIT, respectively. He suffered from cachexia and frequent bleeding as a result of tumor progression.

Overall, myelotoxicity exceeding grade 3 was not observed (Table 2), and non-hematological toxicity consisted of a grade 2 mucositis in one patient (patient 5). Less severe myelotoxicity was observed as compared with patients treated at the highest dose levels in the previous phase I study without the use of re-infusion of G-CSF-stimulated whole blood (Table 3) (*16*). None of the patients who received up to 54

mCi/m^2 developed more than grade 3 myelotoxicity. The MTD in the previous study was established at 27 mCi/m^2, with grade 4 myelotoxicity in two of three patients receiving 41 mCi/m^2. Introduction of this procedure allowed increase of the maximum tolerated bone marrow doses from 0.9 ± 0.2 Gy to 2.5 ± 0.4 Gy, and an increase of the MTD from 27 mCi/m^2 to at least 54 mCi/m^2 (Table 3).

Stable disease was observed in five patients, ranging from 3-5 mo, and still ongoing in one patient (patient 9). No partial or complete responses were seen.

Figure 1. Whole body scan (patient 8) acquired at 72 h after administration of ^{186}Re-cMAb U36. At the site of recurrence in the left oropharynx, accumulation of ^{186}Re-cMAb U36 is clearly visible. Except for blood pool activity, a more or less homogeneous distribution of ^{186}Re-cMAb U36 is seen for the remainder of the whole body.

Table 2. Dose levels, BM dosimetry, myelotoxicity, and response after ^{186}Re-cMAb U36 RIT with re-infusion of G-CSF-stimulated unprocessed whole blood.

Patient	Dose level	Total administered dose	BM dose	Platelet nadir	Platelets <50 x 10^9/L	WBC nadir	Granulocyte nadir	Granulocytes <1.0 x 10^9/L	pRBC transfusions	Response	Duration of response
	(mCi/m^2)	(mCi)	(Gy)	(x 10^9/L)	(no. of days)	(x 10^9/L)	(x 10^9/L)	(no. of days)			(mo)
1	27	46	1.0	144		8.9	6.2		1 x 2	PD	
2	27	38	0.8	206		5.4	4.6			PD	
3	27	59	1.0	201		4.5	3.1			SD	3
4	41	78	1.8	25	5	2.0	1.4		1 x 3[a]	PD	
5	41	70	1.7	67		1.7	0.8	11		SD	5
6	41	76	1.7	59		1.7	2.0		2 x 3	PD	
7	54	81	2.6	21	21	1.5	0.9	4		SD	3
8	54	92	2.8	33	13	1.6	0.9	2		SD	4
9	54	97	2.1	60		2.0	0.8	7		SD	> 5[b]

Abbreviations: BM, bone marrow; pRBC, packed red blood cells; PD, progressive disease; SD, stable disease; [a]Patient 4 also received a transfusion of platelets. [b]stable disease ongoing.

Table 3 Comparison of mean bone marrow (BM) dose and maximum hematological toxicity grade after RIT with ^{186}Re-cMAb U36 in patients treated without re-infusion of G-CSF-stimulated whole blood and patients treated with re-infusion of G-CSF-stimulated whole blood[a]

Patients treated without whole blood support				Patients treated with whole blood support			
Activity level (mCi/m^2)	**No. of patients**	**Mean BM dose (Gy)**	**Maximum hematogical toxicity grade**	**Activity level (mCi/m^2)**	**No. of patients**	**Mean BM dose (Gy)**	**Maximum hematogical toxicity grade**
11	4	0.3 ± 0.1	1	11	-	-	-
27	6	0.9 ± 0.2	3	27	3	0.9 ± 0.1	0
41	3	1.1 ± 0.04	4	41	3	1.7 ± 0.1	3
54	-	-	-	54	3	2.5 ± 0.4	3

[a]Patients treated without re-infusion of G-CSF-stimulated whole blood (left, previous phase I trial described in reference 16), *versus* patients treated with re-infusion of G-CSF-stimulated whole blood in the current study (right). The number of patients treated at each dose level is indicated, as well as the mean bone marrow dose per dose level and corresponding SD.

Discussion

In this study, re-infusion of G-CSF-stimulated unprocessed whole blood was used for dose intensification of RIT with ^{186}Re-cMAb U36. As in chemotherapy, myelotoxicity, in general, is dose-limiting in RIT studies. Whereas after chemotherapy myelotoxicity becomes manifest within a few days, the nadir of platelets and granulocytes after RIT occurs at 4-6 wk. This might allow blood stem cells, if re-infused at a timepoint at which an acceptable low radiation level is present in blood, to home and proliferate in the bone marrow before myelotoxicity becomes manifest, thereby reducing the severity of myelotoxicity.

At the current stage of development, re-infusion of G-CSF-stimulated whole blood should be performed not more than 72 h after harvesting in order to guarantee the viability of the blood stem cells (*9,10*). The effective half-life of ^{186}Re-cMAb U36 is 37 h (*16*), which means that at the time of re-infusion radiation damage to circulating blood stem cells can still occur.

This preliminary phase I study showed improved potential of RIT with ^{186}Re-cMAb U36 in comparison with a previous study where no re-infusion of G-CSF-stimulated unprocessed whole blood was performed. In the current study, patients tolerated higher total injected doses, and higher doses delivered to the bone marrow were tolerated. Stable disease was more frequently observed in the present study. In total, five of nine patients showed stable disease, and all three patients treated at the highest dose level showed stable disease. In the previous study only one of thirteen patients showed stable disease. The only responder had been treated at the highest dose level. The observation of stabilization of disease in patients treated in the current study offers opportunities for further development of RIT in an adjuvant setting, since antibody uptake in small-volume tumors is found to be higher than uptake in large tumors (*22,23*).

An aspect for further improvement is the storage time of whole blood, which is currently limited to 72 h. To increase the clinical applicability, as for RIT with radiolabeled MAbs with a relatively long effective half-life in blood, this storage time should be extended. Encouraging results with new storage mediums have shown preservation of clonogenic capacity of blood stem cells in whole blood for at least 7 days (*24*), which could allow re-infusion at a later, more suitable time-point after RIT, at which remaining radiation levels in blood have been further decreased. For leucopheresis or bone marrow transplantation, this problem of storage time does not play a role. However, both procedures require filtration techniques, cryopreservation, and expensive laboratory facilities. Re-infusion of G-CSF-stimulated unprocessed whole blood can be performed in almost every institution, and at considerably lower

costs.

In conclusion, this study showed that a doubling of the MTD of ^{186}Re-cMAb U36 could be achieved using re-infusion of G-CSF-stimulated unprocessed whole blood. Our results indicated that bone marrow doses up to 2.8 Gy could be reached without development of dose-limiting myelotoxicity or nonhematological toxicity. In five of nine patients with recurrent disease for whom no curative options were available, stabilization of disease was observed.

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5

Safety, biodistribution, pharmacokinetics, and immunogenicity of ^{99m}Tc-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with squamous cell carcinoma of the head and neck

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Abstract

Previous studies have shown the potential of murine and chimeric anti-CD44v6 monoclonal antibodies (MAbs) for radioimmunotherapy (RIT) of head and neck squamous cell carcinoma (HNSCC). A limitation of these MAbs, however, appeared to be their immunogenicity. Therefore, humanized monoclonal antibody BIWA 4 (bivatuzumab), with an intermediate affinity for CD44v6, was recently selected. As a prelude to RIT, we evaluated the safety, tumor-targeting potential, pharmacokinetics, and immunogenicity of technetium-99m-labeled BIWA 4 in patients undergoing operations for primary HNSCC in this study.

Ten patients were treated at BIWA 4 dose levels of 25 mg (*n*=3), 50 mg (*n*=4), and 100 mg (*n*=3). Patients received 2 mg of 750 MBq ^{99m}Tc-BIWA 4, together with 23, 48, and 98 mg unlabeled BIWA 4, respectively. Radioimmunoscintigraphy (RIS) was performed within 1 h and after 21 h, and patients underwent surgery at 48 h after injection. Biodistribution of ^{99m}Tc-BIWA 4 was evaluated by radioactivity measurements in blood, bone marrow, and in biopsies of the surgical specimen obtained 48 h after injection. BIWA 4 concentration in blood was assessed by ELISA and high performance liquid chromatography and related to soluble CD44v6 levels in serum samples. The development of human-anti-human antibody (HAHA) responses was determined.

Administration of ^{99m}Tc-BIWA 4 was well tolerated by all patients and no HAHA responses were observed. A mean $t_{1/2}$ in plasma of 54.8 ± 11.5, 76.1 ± 21.8, and 68.5 ± 21.2 h was found for the 25, 50, and 100 mg dose group, respectively. No complex formation of BIWA 4 with soluble CD44v6 in blood was observed. RIS showed targeting of primary tumors and lymph node metastases in eight of ten and one of five patients, respectively. The highest tumor uptake and tumor to nontumor ratios were observed for the 50 mg dose group. Tumor uptake was 12.9 ± 5.9, 26.2 ± 3.1, and 15.4 ± 1.9 % of the injected dose (ID)/kg for the 25, 50, and 100 mg dose group, respectively, while the tumor to bone marrow ratios for these groups were 1.7 ± 0.5, 3.2 ± 1.1, and 2.0 ± 0.6, respectively.

Conclusion: ^{99m}Tc-BIWA 4 can safely be administered to patients with HNSCC, with absence of detectable HAHA responses. The 50 mg dose level showed the highest tumor uptake and tumor to nontumor ratios. These findings support the use of BIWA 4 for RIT studies with HNSCC.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) accounts for approximately 5% of all newly diagnosed malignancies in Northwestern Europe and the United States, and the worldwide incidence is 500,000 cases each year (*1*). Despite improvements in locoregional treatment by surgery and radiotherapy for advanced stages III and IV (70%), the failure rate, either locally or at distant sites, is still high. An effective systemic adjuvant treatment, therefore, is needed to improve survival in this patient group. Among the novel approaches for selective treatment is the use of monoclonal antibodies (MAbs) conjugated with radionuclides. Radioimmunotherapy (RIT) has shown efficacy in treatment of hematological malignancies (*2,3*).

These successes have motivated us to apply RIT in HNSCC. In the selection of a suitable target antigen in HNSCC, CD44 splice variants containing the v6 domain appeared to be the most promising thus far (*4,5*). The CD44 protein family consists of isoforms, encoded by standard exons and up to nine alternatively spliced variant exons (v2-v10), which are expressed in a tissue-specific way. Soluble v6-containing CD44 (sCD44v6) fragments have been detected in the blood of cancer patients, as well as of healthy individuals (*6*).

In the past decade, the development of CD44v6-directed RIT in HNSCC has shown encouraging progress. For this purpose two murine MAbs (mMAbs) became available designated U36 and BIWA 1. Although both MAbs target the same CD44v6 antigen, BIWA 1 binds to a different epitope with 35-fold higher affinity (*7*). At a later stage, a chimeric derivative of U36 (cMAb U36) also became available. Extensive evaluation of mMAb U36, cMAb U36 and BIWA 1 in HNSCC patients revealed selective tumor targeting and high tumor uptake (*8-10*).

Although not the primary aim in phase I studies, promising antitumor effects were observed in clinical phase I RIT studies (*11,12*). Unfortunately, BIWA 1 showed complex formation with sCD44v6 present in the blood and heterogeneous uptake throughout the tumor (*10*). These phenomena might be related to the high affinity of this MAb. In addition, for all three MAbs a high incidence of human-anti-mouse or human-anti-chimeric antibody responses (HAMA and HACA, respectively) was observed.

Formation of HAMA or HACA should be eliminated to prevent allergic reactions and rapid clearance of the radioimmunoconjugate, especially when multiple administrations of the radioimmunoconjugate are anticipated. The engineering of humanized MAbs (hMAbs), being almost completely human apart from a small part of the antigen-binding sites became the next approach to deal with this problem.

Several studies showed decreased immunogenicity of hMAbs in comparison with their murine counterparts (*13-15*).

The high immunogenicity of the previously tested MAbs led us to construct two humanized derivatives of BIWA 1, designated BIWA 4 and BIWA 8. Together with mMAb U36 and BIWA 1, these new antibodies were evaluated for biodistribution and efficacy in RIT using nude mice bearing HNSCC xenografts. Remarkably, the lower-affinity MAbs showed a superior tumor targeting capacity in HNSCC-bearing nude mice (*7*). For this reason the intermediate affinity hMAb BIWA 4 rather than the high affinity hMAb BIWA 8 was selected as candidate for further clinical development.

As prelude to RIT, in the present study ^{99m}Tc-BIWA 4 was evaluated for its safety, biodistribution, pharmacokinetics, and immunogenicity in patients who underwent surgery for primary HNSCC. These parameters were evaluated at three different BIWA 4 dose levels in order to select an optimal dose level for future RIT studies.

Patients and methods

Patient eligibility

Ten patients with histologically proven HNSCC were included in this study, while a biopsy of their primary tumor had to show expression of CD44v6 in at least 50% of the cells (semiquantitatively determined). Their characteristics are shown in Table 1. Patients who participated were planned to undergo resection of the primary tumor and unilateral or bilateral neck dissection. Other eligibility criteria were age between 18 and 80 years, and a Karnofsky performance status of 70 or greater. Exclusion criteria were a life-threatening infection, allergic diathesis, serious cardiac disease, alimentary or contact allergy, severe atopy or allergy, pregnancy, and chemotherapy or radiotherapy within four weeks prior to inclusion. Patients were excluded if their white blood cell count was less than 3500/μL, if platelets were lower than 150,000/μL, if the serum creatinine concentration was greater than 150 μmol/mL, or the bilirubin level was greater than 40 μmol/mL. The study was approved by the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands). All patients gave signed informed consent after receiving a thorough written and oral explanation of the study.

BIWA 4 (bivatuzumab) and CD44v6

The parental MAb of BIWA 4, BIWA 1 (Boehringer Ingelheim, Vienna, Austria) was generated by immunizing BALB/c mice with glutathione S-transferase fusion protein containing the human CD44 domains v3-v10. The epitope recognized by BIWA 1 has been mapped to amino acids 360-370 in domain v6 of CD44 (numbering according to Kugelman *et al.*) (*16*). Homogeneous expression of v6-containing CD44 has been observed in squamous cell carcinoma of the head and neck, lung, skin, esophagus, and cervix, while heterogeneous expression was found in adenocarcinomas of the breast, colon, lung, pancreas, and stomach (*5*). In normal tissues, expression has been found in epithelial tissues as oral mucosa, skin, breast and prostate myoepithelium, and bronchial epithelium (*17*).

TABLE 1. PATIENT CHARACTERISTICS

Patient no.	Age	Sex	Primary tumor site	Stage	BIWA 4 dose	Visualization of primary tumor	Visualization of lymph node metastasis
	(y)				(mg)		
1	37	M	parotid gland	T4N0	25	Yes	NA
2	45	M	oropharynx, tonsillolingual sulcus	T2N1	25	Yes*	No
3	55	F	oral cavity, retromolar area	T4N1	25	Yes	Yes
4	78	F	oral cavity, buccal mucosa	T3N1	50	Yes	No
5	38	M	oral cavity, inferior alveolar process	T4N0	50	No	NA
6	67	F	larynx, supraglottis	T4N2c	50	Yes	No
7	51	M	oropharynx, tonsil	T3N1	50	Yes	No
8	38	M	oral cavity, lateral tongue	T2N0	100	No	NA
9	72	F	oral cavity, floor of mouth	T2N0	100	Yes*	NA
10	66	F	oropharynx, base of tongue	T2N0	100	Yes*	NA

* Tumor or lymph node metastasis only detected by SPECT imaging. Abbreviations: M, male; F, female; NA, not applicable.

Generation, production, and characterization of BIWA 4 has been described previously (*7*). Humanized versions of the BIWA 1 heavy and light chain variable regions generated by complementarity-determining regions (CDR) grafting were cloned in front of the immunoglobulin constant regions of the above mentioned expression vectors, resulting in BIWA 4. Clinical grade BIWA 4 was supplied by Boehringer Ingelheim, Germany.

It was anticipated that the optimal MAb dose for future RIT would be in the range of 25-100 mg. The most optimal dose should result in a high relative tumor uptake, high tumor to nontumor ratios, and a homogeneous MAb distribution throughout the tumor. With respect to the latter, an increased MAb dose will result in a more homogeneous distribution (*8,10*). Three patients were intended to be treated at each dose level of 25, 50, and 100 mg, respectively. Since no BIWA 4 tumor uptake data were obtained in one patient of the 50 mg dose group (patient 5), an extra patient was included at this dose level. All other data obtained from patient 5 were used for analysis.

Radiolabeling and quality controls

Radiolabeling of BIWA 4 with ^{99m}Tc was performed using the chelate *S*-benzoyl-mercaptoacetyltriglycine (MAG3) as described previously (*18*). Both ^{99m}Tc and the chelate were obtained from Mallinckrodt, Inc. (Petten, The Netherlands). In short, after synthesis of ^{99m}Tc-MAG3 an esterification with tetrafluorophenol was performed. Subsequently, the ester was purified and conjugated to BIWA 4. Radiolabeled BIWA 4 was purified on a PD10 column (Pharmacia-Biotech, Woerden, The Netherlands) with 0.9% NaCl as eluent. The conjugates were filter sterilized. Radiochemical purity of the conjugates was assessed by thin-layer chromatography (TLC) with a mean value of 98.4 % (range 96.9 – 99.1 %). The immunoreactive fraction of ^{99m}Tc-BIWA 4 was determined by linear extrapolation to conditions representing infinite antigen excess using a modified Lineweaver Burk plot (*18*). A mean immunoreactive fraction of 94.7 ± 5.6 % was found for eight of ten patients. For the other 2 patients these data are missing due to technical difficulties.

Study design

Patients' histories and physical conditions were examined, and routine laboratory analyses were performed, including serum electrolytes, hepatic enzymes, thyroid and renal functions, and urine. Complete blood cell and platelet counts, an electrocardiogram, and a chest radiograph were obtained. Before surgery, patients underwent imaging by CT or MRI, as well as a panendoscopy. BIWA 4 was administered intravenously in 5 min at a dose of 2 mg and 20 mCi (750 MBq) ^{99m}Tc-BIWA 4, with 23, 48, or 98 mg unlabeled BIWA 4, respectively. Vital signs (blood pressure, pulse rate, breathing rate, and temperature) were recorded just prior to administration, and after 10, 60, and 120 min. Scintigraphy studies were performed using a large field-of-view gamma camera (Dual Head Genesys Imaging System, ADAC Laboratories, Milpitas, CA) equipped with a low-energy high-resolution parallel-hole collimator and connected to a computer system (Pegasys, ADAC Laboratories, Milpitas, CA). Camera quality control measures were taken at each imaging time. Planar anterior and posterior whole-body scans were obtained within 1 h after administration of ^{99m}Tc-BIWA 4, and after 21 h. Lateral, anterior, and posterior planar images and SPECT images of the head and neck region were acquired at 21 h after administration. The results of the scintigraphy and CT/MRI studies were each scored by one experienced examiner. The two examiners were blinded to the results of other examinations and the histopathological outcome of the surgical specimen. Surgery was performed 48 h after administration of ^{99m}Tc-BIWA 4. From the surgical specimen, biopsies were taken of tumor tissue, lymph node metastases, and various normal tissues (fat, muscle, skin). Blood, bone, and bone

marrow were obtained immediately before surgery under general anesthesia. Tissue and blood samples were weighed, and the amount of ^{99m}Tc was measured in a gamma-well counter (1470 Wizzard; Wallac, Turku, Finland). Tumor to nontumor values were calculated using matched uptake values of one patient. After counting, biopsies were evaluated by histopathological analysis.

Pharmacokinetics

Blood samples were taken from the opposite antecubital vein preinfusion, and at 5, 10, and 30 min, and 1, 2, 4, 16, 24, 48, 72, and 144 h after administration. Urine was collected from 0-4, 4-8, 8-12, 12-24, 12-24, and 24-48 h after administration to determine excretion of radioactivity. Plasma samples were prepared for determination of the concentration of (immunoreactive) BIWA 4 by validated ELISA method, essentially as described previously (*10*). In addition, whole-blood and serum samples were prepared and counted together with the urine samples in a gamma-counter (1470 Wizard; Wallac, Turku, Finland), and radioactivity levels were expressed as percentage of the injected dose per kg tissue (%ID/kg). Background activity and decay were corrected for and the %ID/kg was determined by comparison with an aliquot retained from the conjugate preparation for injection.

Non-compartmental pharmacokinetic parameters were calculated using WinNonlin 3.1 Professional (Pharsight Corporation, Mountain View, CA). Size exclusion chromatography (silica-based gel filtration high-performance liquid-chromatography, HPLC) was applied to assess the percentage of radiolabeled free BIWA 4 and complexed BIWA 4, essentially as described previously (*10*). Levels of sCD44v6 were assessed in serum samples obtained before administration of BIWA 4, and after 21, 48, and 144 h and at 6 wk. A commercially available sandwich type ELISA was used (Bender MedSystems, Vienna, Austria) (*19*).

Human-anti-BIWA 4 response

Evaluation of the immunogenicity of the BIWA 4 radioimmunoconjugate was conducted by determination of human-anti-hMAbs (HAHAs) in serum samples taken prior to administration of ^{99m}Tc-BIWA 4, and 1 and 6 wk after administration, using a validated bridging ELISA. In short, BIWA 4 antibodies labeled with the chelator MAG3 were immobilized onto microtiter plates. Antibodies to MAG3-BIWA 4 present in the serum sample bound simultaneously to the solid phase and to biotinylated MAG3-BIWA 4 that was added at the same time in a fixed amount. Streptavidin coupled to horseradish peroxidase (Boehringer Mannheim, Germany) was used for signal generation by the peroxidase/tetramethyl-benzidine system. A

serum sample was considered HAHA positive only, if all the following criteria were met: 1) The measured titer was above the lower limit of determination/quantification, 2) The titer in the post-injection sample was at least two times higher than in the pre-injection sample, 3) The sample could be serially diluted with a strong correlation between titer and dilution factor, 4) The titer could be blocked by addition of BIWA 4, and 5) The titer could not be blocked by a nonspecific IgG MAb.

Statistical analysis

All mean values reported represent arithmetic means and corresponding standard deviations. Two-sided significance levels were calculated for all parameters, with $P < 0.05$ considered statistically significant.

Results

No serious adverse reactions to ^{99m}Tc-BIWA 4 were observed in any of the patients, and no clinically significant changes in vital signs and blood analyses were noted. One patient (patient 6) developed dyspnea 10 min after administration of ^{99m}Tc-BIWA 4, which was treated with administration of oxygen and steroids. The dyspnea lasted for less than 2 min. No other signs indicating an immunological reaction (rash, fever, hypotension) were observed, and no positive HAHA titer was measured in the serum of this patient. The reaction was attributed to hyperventilation because of mental stress. However, a relation with administration of ^{99m}Tc-BIWA 4 could not be excluded. Another patient (patient 1) required a reintervention directly after primary surgery because of bleeding from the wound. He recovered well, and no relation with the administration of ^{99m}Tc-BIWA 4 was suspected. Patient 10 developed a thrombophlebitis of the external jugular vein 4 days after surgery. A reintervention was performed, and the patient was treated with antibiotics. A relation with the administration of ^{99m}Tc-BIWA 4 appeared unlikely.

Imaging showed mainly blood-pool activity on whole-body scans made directly after administration of ^{99m}Tc-BIWA 4, which had decreased after 21 h, and showed a more or less homogeneous distribution for lungs, liver, spleen, and kidneys, and increased uptake at tumor sites. Planar imaging and SPECT at 21 h after administration showed uptake of ^{99m}Tc-BIWA 4 at the primary tumor site in eight of ten patients. In only one of five patients with histopathologically proven lymph node metastases in the neck, SPECT revealed focal uptake (Figure 1). No selective accumulation was observed at nontumor sites. In one of the patients (patient 5), the primary tumor in the inferior alveolar process was considered too small (< 5 mm) for

detection and no tumor tissue could be obtained for biodistribution analysis. All other data obtained from patient 5 were used for analysis. An extra patient was included at the 50 mg BIWA 4 dose level. The other patient (patient 8), where scintigraphy could not detect the primary tumor, had a tongue carcinoma measuring 40 x 20 mm. Mobility of the tongue during scintigraphy might have influenced image quality, since biopsy of the primary tumor showed normal uptake (15.9 % ID/kg) of ^{99m}Tc-BIWA 4.

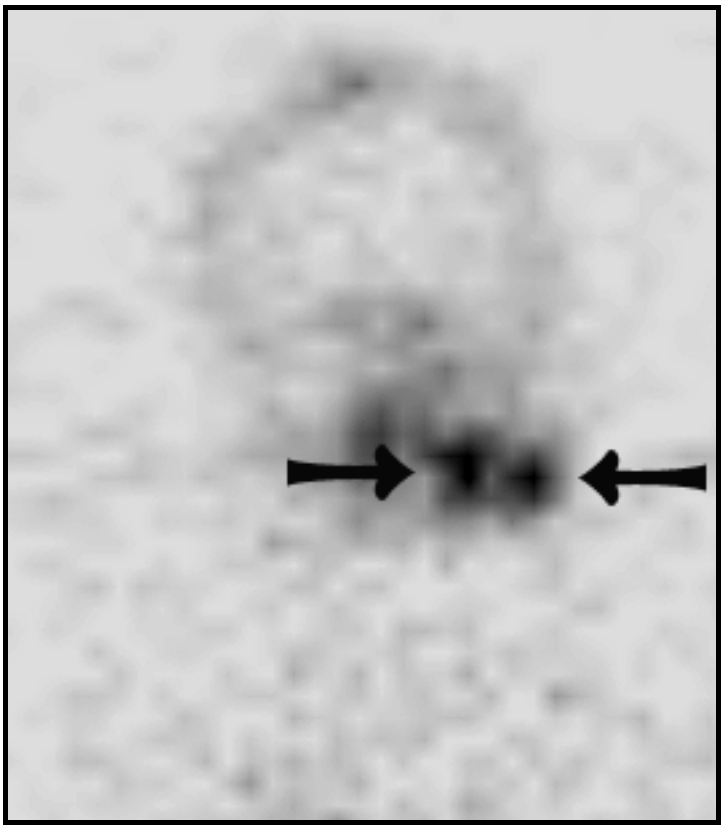

Figure 1. Coronal SPECT image at 21 h after administration of ^{99m}Tc-BIWA 4 of patient with primary oral cavity carcinoma (left arrow) and neck lymph node metastasis in level II (right arrow) (patient 3).

Pharmacokinetic analyses of BIWA 4 in plasma as assessed by ELISA showed a mean $t_{1/2}$ of 54.8 ± 11.5, 76.1 ± 21.8, and 68.5 ± 21.2 h for the 25, 50, and 100 mg dose group, respectively. BIWA 4 concentrations as assessed by this ELISA method were consistent with radioactivity concentrations for samples collected untill 48 h after injection (data not shown). Due to the short half-life of ^{99m}Tc accurate measurements of radioactivity concentrations were not possible at later time points. For six patients with complete 48 h urine collections, the mean amount of radioactivity collected was 10% of the administered dose. Levels of sCD44v6 in

serum did not change after administration of ^{99m}Tc-BIWA 4 for the three different BIWA 4 dose groups (Table 2). HPLC analysis did not reveal complex formation of BIWA 4 with sCD44v6.

Table 2. Soluble CD44v6 in serum before and after administration of hMAb BIWA 4.

hMAb BIWA 4 dose (mg)	25	50	100
Time after administration (h)			
preadministration	203 ± 46	337 ± 73	174 ± 21
21	223 ± 34	301 ± 76	179 ± 9
48	200 ± 41	275 ± 82	157 ± 12
144	188 ± 40	282 ± 104	182 ± 59
6 wk	203 ± 32	310 ± 53	210 ± 18

Levels of soluble CD44v6 in serum (mean values, in ng/mL) at different times before and after administration of ^{99m}Tc-hMAb BIWA 4 as assessed by ELISA.

Uptake results of ^{99m}Tc-BIWA 4 in tumor and nontumor tissue biopsies are summarized in Table 3. The highest tumor uptake was observed in the 50 mg dose group, ranging from 22.6-28.1 %ID/kg. In this dose group the tumor to bone marrow ratio was 3.2 ± 1.1, whereas tumor to bone marrow ratios for the 25 mg and 100 mg dose group were 1.7 ± 0.5, and 2.0 ± 0.6, respectively (Table 4).

None of the patients showed a HAHA response following administration of ^{99m}Tc- BIWA 4.

Table 3. Uptake (%ID/kg) of ^{99m}Tc-BIWA 4 in biopsies obtained from surgical specimens at 48 h after administration.

TISSUES	25 mg BIWA 4	50 mg BIWA 4	100 mg BIWA 4
Tumor	12.9 ± 5.9	26.2 ± 3.1	15.4 ± 1.9
Mucosa	8.6 ± 1.7	10.6 ± 4.6	9.1 ± 3.2
Positive lymph node	6.1 ± 3.2	7.6 ± 2.9	NA
Normal lymph node	2.9 ± 2.8	1.6 ± 0.5	1.8 *
Muscle	2.0 ± 0.4	1.4 ± 0.2	1.2 ± 0.3
Fat	2.6 ± 0.4	1.9 ± 1.2	1.6 ± 0.4
Bone	1.3 ± 0.8	1.6 ± 1.1	1.1 ± 0.1
Glandular tissue	5.2 ± 2.3	4.9 ± 0.6	5.3 *
Vein	2.6 ± 0.1	2.5 ± 1.2	2.9 *
Blood	8.7 ± 1.9	8.9 ± 2.0	8.8 ± 1.7
Total bone marrow aspiration	7.2 ± 1.5	8.5 ± 2.1	8.1 ± 1.9
Supernatant bone marrow aspiration	14.4 ± 2.2	13.5 ± 2.7	13.9 ± 1.1
Sediment bone marrow aspiration	3.6 ± 1.1	4.1 ± 1.9	5.7 ± 2.4

- Samples were available from only one patient.

Discussion

Administrations of ^{99m}Tc-BIWA 4 were well tolerated by all patients and no adverse-effects related to BIWA 4 were noticed. Since none of the patients showed a HAHA response, it can be concluded that the immunogenicity of BIWA 4 is less than that of the parental murine BIWA 1, as well as that of both murine and chimeric MAb U36. These findings support the feasibility of a multiple administration regimen of BIWA 4 in future RIT studies, and might indicate a reduced risk of allergic reactions following administration. In the meantime, 18 patients more have been treated with 50 mg radiolabeled BIWA 4 in 2 parallel radioimmunoscintigraphy-biodistribution studies with non-small-cell lung cancer and breast cancer patients (data not published). In none of these additional patients HAHA responses were observed. This result compares favourably with those found for cMAb U36 (40% HACAs) (*11*), and the parental mouse MAb BIWA 1 (90% HAMAs) (*10*).

Table 4. Tumor to nontumor ratios of ^{99m}Tc-BIWA 4 in biopsies obtained from surgical specimens 48 hours after administration.

TISSUES	25 mg BIWA 4	50 mg BIWA 4	100 mg BIWA 4
Mucosa	1.9 ± 0.3	3.1 ± 1.6	1.9 ± 0.7
Normal lymph node	6.2 ± 3.9	18.0 ± 5.8	7.5 *
Muscle	6.5 ± 2.7	19.8 ± 6.1	13.7 ± 3.2
Fat	4.9 ± 2.0	23.2 ± 16.6	9.5 ± 0.8
Bone	17.2 ± 16.3	11.6 *	13.7 ± 2.3
Glandular tissue	3.4 ± 1.3	6.1 ± 0.9	2.5 *
Vein	4.1 ± 2.5	12.6 ± 6.0	4.5 *
Blood	1.4 ± 0.5	2.9 ± 0.9	1.8 ± 0.5
Total bone marrow aspiration	1.7 ± 0.5	3.2 ± 1.1	2.0 ± 0.6
Supernatant bone marrow aspiration	0.9 ± 0.3	2.1 ± 0.7	1.1 ± 0.2
Sediment bone marrow aspiration	3.5 ± 1.1	7.2 ± 3.2	3.2 ± 1.6

* Samples were available from only one patient.

Humanization of other MAbs has been performed with variable success. For instance, hMAb M195 elicited no immune response in a study group of 14 patients compared with 37% HAMAs found with mMAb M195 (*13,20*). Furthermore, hMAb BrE-3 showed 14% HAHAs (*14*), compared with mMAb BrE-3, which showed immunogenicity in 47-83% of patients (*21*). On the other hand, hMAb A33, elicited HAHAs in 26 of 41 patients (*15*). Although significantly lower as compared to its parental mMAb A33, for which HAMAs were found in all treated patients (*22*), this example illustrates that humanization not always solves the problem of immunogenicity.

Scintigraphy identified eight of ten primary tumors and lymph node metastases in only one of five patients. Size and localization of the primary tumors and lymph node metastases might have played a role in causing poor imaging results. Another explanation is that the short physical half-life of ^{99m}Tc is not well-matched with the relatively long plasma $t_{1/2}$ of BIWA 4, the latter causing a relatively high blood-pool activity at the time of imaging, hampering delineation of tumors and lymph node metastases.

In an earlier biodistribution study with 2, 12, and 52 mg ^{99m}Tc-BIWA 1, levels of sCD44v6 in serum apparently fell during the first week after administration, and extensive complex formation of BIWA 1 with sCD44v6 was observed, especially at the 2 mg dose level. Moreover, a heterogeneous distribution of BIWA 1 throughout the tumor was observed (*10*). Both phenomena were attributed to the high affinity of BIWA 1 to CD44v6. In the current study, no changes in serum levels of sCD44v6 were observed after administration for three different BIWA 4 dose levels. Moreover, no complex formation of BIWA 4 with sCD44v6 was observed. Consistent with the findings from previous biodistribution studies with nude mice bearing HNSCC xenografts (*7*), tumor uptake values of the intermediate affinity BIWA 4 were high, higher than for the high affinity BIWA 1, and similar to the uptake values of the low affinity mMAb U36. While tumor uptake at 48 h after administration was 26.2 ± 3.1 %ID/kg for BIWA 4 at the 50 mg dose level, uptake of BIWA 1 and mMAb U36 at this dose level was 8.0 ± 2.8 %ID/kg and 20.4 ± 12.4 %ID/kg, respectively.

Apparently, BIWA 4 has a higher avidity for membrane-bound v6-containing CD44 variants than for soluble CD44v6 variants. The high density of CD44v6 on tumor cell membranes may allow bivalent binding, whereas bivalent binding to soluble CD44v6 variants is not possible as each molecule contains only a single epitope. Furthermore, v6-containing CD44 variants present on tumor cell membranes may have better interaction with BIWA 4 than the variants present in the circulation because of conformational differences. Reduced affinity for binding to circulating antigen in comparison to immobilized antigen has also been described for other antibodies, including MAbs directed against carcinoembryonic antigen (*23,24*).

In conclusion, this study shows that ^{99m}Tc-BIWA 4 can safely be administered, selectively targets HNSCC with high tumor uptake, and does not induce detectable HAHA responses after a single administration. The 50 mg dose seems to be optimal since it shows high tumor uptake and a favourable tumor to bone marrow ratio.

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6

Clinical significance of micrometastatic cells detected by E48 (Ly-6D) reverse transcriptase-polymerase chain reaction in bone marrow of head and neck cancer patients.

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Submitted

Abstract

Despite improvements in locoregional treatment of squamous cell carcinoma of the head and neck (HNSCC), local and distant failure rates remain high. A putative prognostic indicator in HNSCC and other epithelial malignancies, enabling more accurate staging and selection of patients for whom adjuvant therapy is recommended, is the presence of micrometastatic cells in bone marrow. The gene encoding the E48 antigen is selectively expressed by HNSCC, and the detection of E48 transcripts in bone marrow by reverse transcriptase-polymerase chain reaction (RT-PCR), presumably represents the presence of micrometastatic cells. The purpose of this study was to determine the association between the presence of micrometastatic cells in bone marrow of HNSCC patients and clinical outcome.

A total of 162 patients treated surgically for primary HNSCC underwent a single bone marrow aspiration from the upper iliac crest for detection of micrometastatic cells using E48 RT-PCR. In total 139 patients were evaluable. The major statistical endpoint were disease-free and distant metastases-free survival.

E48 RT-PCR indicated the presence of bone marrow micrometastatic cells in 56/139 (40%) of the patients. Presence of micrometastatic cells had no significant influence on disease-free survival or distant metastases-free survival for the whole group of patients ($P = 0.1460$ and $P = 0.2912$, respectively). For patients with ≥ 2 lymph node metastases, however, the presence of micrometastatic cells was associated with a poor distant metastases-free survival ($P = 0.0210$).

Conclusion: the presence of micrometastatic cells in bone marrow of HNSCC patients with ≥ 2 lymph node metastases is correlated with a poor distant metastases-free survival. In this subgroup of HNSCC patients, the E48 RT-PCR can be a valuable tool to identify patients who are at increased risk for development of distant metastases, and therefore might benefit from adjuvant systemic therapy.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents ~95% of head and neck tumors. The estimated incidence worldwide in 2000 was approximately 551,000 cases, and 217,000 died of the disease (*1*). About one third of the patients presents with early stage (I and II) disease, while two third of the patients present with advanced stage (III and IV) disease (*2*). Initial therapy of HNSCC is surgery and/or radiotherapy. While early stages I and II generally have a good prognosis, the more advanced stages have a high failure rate. Despite improvements in locoregional control for patients with advanced HNSCC, the overall survival rates have not significantly improved over the last 25 years, as the incidence of metastases at distant sites increased (*3*).

In large series of patients, the development of distant metastases, generally` within two years after locoregional treatment, ranges between 5-25%, whereas in subgroups of patients with advanced disease the incidence of distant metastases can be much higher (*4-9*). The most important prognostic indicator for relapse of HNSCC, either locoregionally or at distant sites, is the presence of metastatic spread to lymph nodes in the neck. When multiple lymph node metastases are present, the incidence of distant metastases can be as high as 50% (*4,6,7,9,10*).

The presence of small tumor deposits or even single metastatic tumor cells in bone marrow of patients with various types of epithelial cancers can be detected by immunocytochemistry and molecular methods, notably the polymerase chain reaction (PCR). Both techniques mainly use tissue-specific marker antigens such as cytokeratins, since these are abundantly expressed in the majority of epithelial tumors and homogeneously among the cells of these tumors (*11*). The clinical relevance of micrometastatic cells in bone marrow has been illustrated convincingly for breast and colorectal cancer patients, in whom their presence correlates with poor prognosis (*11-14*). The early detection of these micrometastatic cells could thus contribute to a more accurate staging and the identification of patients who might benefit from adjuvant therapy.

Detection of single tumor cells in bone marrow of HNSCC patients with immunocytochemical techniques using monoclonal antibodies directed against cytokeratins seems a feasible approach (*15,16*). These immunocytochemical methods are difficult to standardize, and many researchers use subjective morphological criteria to assign immunostained cells as cancer cells (*17*). RT-PCR methods are an alternative approach. For detection of micrometastatic cells in the bone marrow of HNSCC patients we exploited the E48 antigen. The gene encoding the E48 antigen is selectively expressed in HNSCC, and previously we showed that E48 RNA transcripts

can be detected in bone marrow aspirates of 35% of HNSCC patients, suggesting the presence of micrometastatic cells, whereas samples of noncancer controls were negative (*18*).

In this study, the clinical significance of micrometastatic cells in bone marrow as detected by E48 RT-PCR at initial work-up is assessed in HNSCC patients who were treated with primary surgery and postoperative radiotherapy and then followed for a median period of 49 months.

Patients and methods

Patients

From December 1995 to August 2000, a total of 162 patients underwent bone marrow aspiration just before primary surgical treatment for HNSCC. Eligible were patients with a primary HNSCC tumor scheduled for surgery and no history of malignancy in the past. Criteria for analysis were histological tumor-free margins and good quality RNA isolated from the bone marrow sample. Based on these criteria for analysis, a total of 23 patients were excluded (in 11 patients the surgical margins were not histogically tumor-free, and in 12 cases the RNA quality was poor). Classification and staging of the primary tumor was performed according to the TNM system of the International Union Against Cancer (Union Internationale Contre le Cancer, UICC) (*19*). Postoperative radiotherapy was given to patients depending on stage and histopathological evaluation. After primary treatment patients were followed regularly at the outpatient clinic. The study was approved by the Institutional Review Board of the VU University Medical Center (Amsterdam, The Netherlands), and all patients signed informed consent.

Preparation of bone marrow samples and RNA isolation

Just prior to surgery, a bone marrow sample (2-5 ml) was obtained under general anaesthesia from the upper iliac crest by needle aspiration on one side and stored in heparin-treated tubes. Samples were diluted 1:1 in RPMI 1640. After centrifugation at 220 x *g* for 10 min, the erythrocytes in the pellet were lysed in 5 ml of lysis buffer containing 160 mM $KHCO_3$, and 0.1 mM EDTA (pH 7.4) and incubated at 4° C for 15 min while the solution was mixed occasionally by inverting the tube. Nucleated cells were pelleted by centrifugation at 220 x *g* for 10 min, washed with 10 ml of RPMI 1640, and centrifuged at 220 x *g* for 5 min. Cells were resuspended in 1 ml of RPMI, transferred to a 1.5-ml microcentrifuge tube, and centrifuged at 12,000 x *g* for 1 min. The pellet was dissolved in 1.0 ml of RNAzol-B (Campro Scientific BV, Veenendaal, the Netherlands) and mixed. After the addition of 100 µl of chloroform, the sample was mixed vigorously for 15 s and put on ice for

5 min. After centrifugation at 12,000 x *g* for 15 min at 4°C, the aqueous phase was transferred to another microcentrifuge tube, while the RNA was precipitated by addition of an equal volume of isopropanol, and stored until use at -20°C. Just before use, RNA was pelleted by centrifugation at 12,000 x *g* for 30 min at 4°C. The pellet was washed by vortexing in 70% ethanol, centrifuged at 12,000 x *g* for 5 min at 4°C, and dissolved in 25-100 µl of RNAse-free H_2O and incubated at 65°C for 15 min. The amount of RNA was calculated from the absorbance at 260 nm, and 5 µg was assayed routinely.

E48 RT-PCR and quality assurance

The standardized RT-PCR technique for detection of E48 RNA transcripts in bone marrow has been described previously (*18*). In short, 5 µg of total RNA was reverse transcribed in 20 µl and on 4 x 5 µl RT-product a PCR was performed (quadruplicate analysis). Amplimers were electrophorized on an agarose gel, blotted and hybridized with the E48 cDNA as a probe. A serial dilution of RNA from the E48 expressing cell line UM-SCC-22A, ranging from 50, 15, 5, 1.5, to 0.5 pg, was run in parallel in each experiment for calibration, as described previously (*20*). All primers, probes and buffers were prepared in a laboratory that is isolated from sample preparation and PCR product analysis. RNA isolation, cDNA synthesis, and preparation of PCR reactions were performed in a pre-PCR laboratory. To prevent amplimer carryover contamination, all materials and reagents were transported in a one-way direction from the pre-PCR laboratories to the PCR laboratory. Sample-to-sample carryover contamination was further avoided by using different pipet sets and filter tips (Greiner Bio-One, Kremsmünster, Austria). Preparations without RNA template were used as negative RT-PCR control. In addition, a blotting control was loaded on the agarose gels consisting of dilutions of EcoRI cut plasmid DNA containing the E48 cDNA (*21*). Autoradiography was standardized using the blotting control. An assay was considered reliable when at least one of four signals was seen at 0.5 pg UM-SCC-22A RNA. A sample was considered positive when at least one of four PCR reactions was positive. RT-PCR amplification of β2-microglobulin transcripts was used as control for the quality of the RNA. These amplimers were loaded on an agarose gel and stained with ethidiumbromide. Examples are depicted in Figure 1.

Statistical analysis

Differences in frequency distributions of clinical characteristics were analyzed by either Student's t-test or the Chi-square test (for two-by-two tables Fisher's exact test). The major statistical endpoint of the prognostic study was the number of patients remaining free of recurrent disease and distant metastases using Kaplan-Meier life-table analyses and log-rank tests (Mantel-Cox). Time to recurrence or death was measured from the date of primary surgery. Patients who developed a second primary tumor were censored for all outcomes at the incidence date of the second tumor, since development of recurrent disease in such case could not be related to the primary tumor alone. Delayed lymph node metastases that developed in an untreated neck during follow-up were not regarded as recurrence. Statistical analyses were performed with the statistical software package BMPD (*22*). Data summaries and graphical presentations were obtained using SPSS software (*23*). *P* values < 0.05 were considered as significant.

Results

The E48 RT-PCR assay detected E48 transcripts in bone marrow aspirates of 56 of 139 (40%) patients. No significant differences in frequency distributions between the clinical characteristics of patients were seen for the presence or absence of a positive E48 RT-PCR result (Table 1). Of all patients, 37% presented with stage I or II disease, whereas 63% were staged as III or IV. The primary tumors were staged pT1 (16%), pT2 (33%), pT3 (34%), and pT4 (17%). In 60 of 139 (43%) patients, lymph node metastases were detected by histopathological examination of the neck dissection specimen. Extranodal spread in one or more lymph node metastases was noticed in 46 of 60 (77%) patients with lymph node metastases.

The median follow-up of patients was 49 months, ranging from 5 to 87 months. An overview of the type of events during follow-up is given in Table 2. Recurrent disease occurred in 24 of 139 patients, of whom 8 had a local recurrence, 3 patients developed regional recurrent disease, and 13 patients presented with distant metastases during follow-up. A second primary tumor was diagnosed in 14 patients. In 10 patients, who presented at initial diagnosis with a clinically negative neck and did not undergo elective neck dissection, lymph node metastases in the neck occurred during follow-up and they were treated with a delayed neck dissection. None of these patients developed recurrent disease during further follow-up.

Table 1. Clinical characteristics of 139 HNSCC patients, with presence or absence of occult micrometastatic cells as assessed by E48 RT-PCR on bone marrow aspirates.

Characteristic		All Patients	E48 RT-PCR negative		E48 RT-PCR positive		*P* value
		No.	No.	%	No.	%	
All patients		139	83	*60*	56	*40*	
Mean age		58	58		57		0.5129 [a]
Sexe							
	Male	94	56	*60*	38	*40*	1.000 [b]
	Female	45	27	*60*	18	*40*	
Site							
	Oral cavity	84	47	*56*	37	*44*	0.1185 [b]
	Oropharynx	37	23	*62*	14	*38*	
	Hypopharynx	11	10	*91*	1	*9*	
	Larynx	7	3	*43*	4	*57*	
T stage							
	pT1	22	15	*68*	7	*32*	0.6781 [b]
	pT2	46	26	*57*	20	*43*	
	pT3	48	30	*62*	18	*38*	
	pT4	23	12	*52*	11	*48*	
N stage							
	≤ pN2a	92	57	*62*	35	*38*	0.4699 [c]
	≥ pN2b	47	26	*55*	21	*45*	
Stage							
	I/II	52	31	*60*	21	*40*	1.000 [c]
	III/IV	87	52	*60*	35	*40*	
Number of lymph node metastases							
	≤ 1	92	57	*62*	35	*38*	0. 4699 [c]
	≥ 2	47	26	*55*	21	*45*	
Extranodal spread							
	Absent	57	40	*70*	17	*30*	0.1061 [c]
	Present	46	25	*54*	21	*46*	
Postoperative radiotherapy							
	Yes	96	57	*59*	39	*41*	0.9040 [b]
	No	43	26	*60*	17	*40*	

Differences between frequency distributions were calculated with [a]Student's T-test, [b]Chi-square, [c]Fisher's exact test.

Table 2. Events[1] during follow-up and type of recurrent disease for HNSCC patients with or without micrometastatic cells in bone marrow as assessed by E48 RT-PCR at initial work-up.

	E48 RT-PCR		
	Negative	**Positive**	**Total**
Disease free	50	31	81
Death other cause, no recurrent disease	7	3	10
Delayed lymph node metastases	5	5	10
Local recurrence	3	5	8
Regional recurrence	2	1	3
Distant metastases	6	7	13
Second primary tumor	10	4	14
Total	83	56	139

[1] only primary events during follow-up were used for analysis

Presence of micrometastatic cells in bone marrow, as assessed by a positive E48 RT-PCR result, had no significant influence on disease-free survival (Figure 2) or distant metastases-free survival ($P = 0.1460$ and $P = 0.2912$, respectively). Standard risk factors like the presence of multiple lymph node metastases and extranodal spread showed a significantly higher risk for development of recurrent disease ($P < 0.0001$). Based on the number of lymph node metastases, we were able to define a good (≤ 1 lymph node metastases) and a poor prognosis (≥ 2 lymph node metastases) group (Figure 3). For both groups, disease-free survival was not significantly associated with the presence of E48 transcripts in the bone marrow, although a trend is observed in the poor prognosis group ($P = 0.7601$ and $P = 0.0908$, for patients with ≤ 1 lymph node metastasis *versus* patients with ≥ 2 lymph node metastases, respectively). For patients with ≤ 1 lymph node metastasis, no significant relation between a positive E48 RT-PCR result and distant metastases-free survival was found ($P = 0.4215$), but for patients with ≥ 2 lymph node metastases, a positive E48 RT-PCR result was associated with a poor distant metastases-free survival ($P = 0.0210$). Kaplan-Meier life-table analyses are depicted in Figure 4.

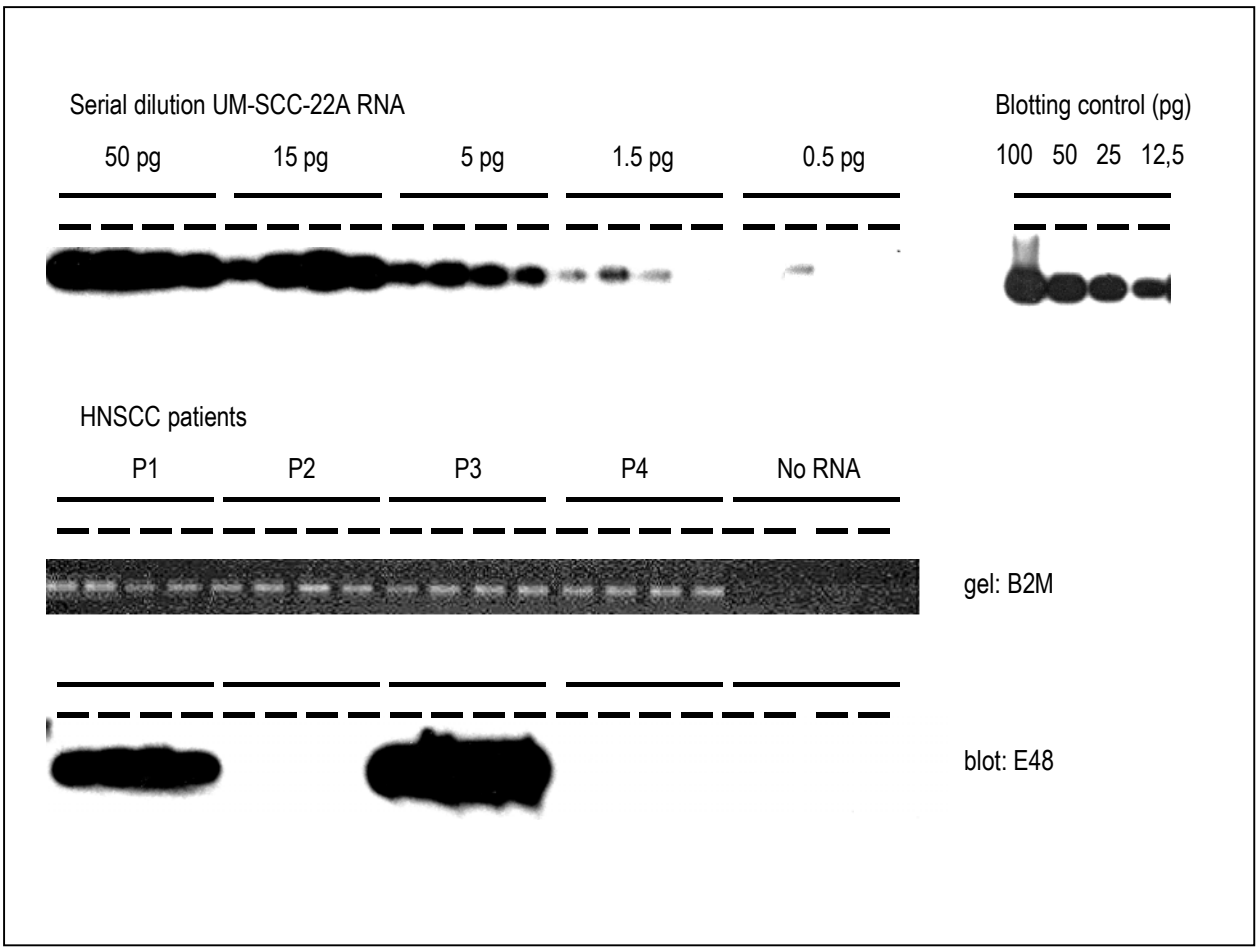
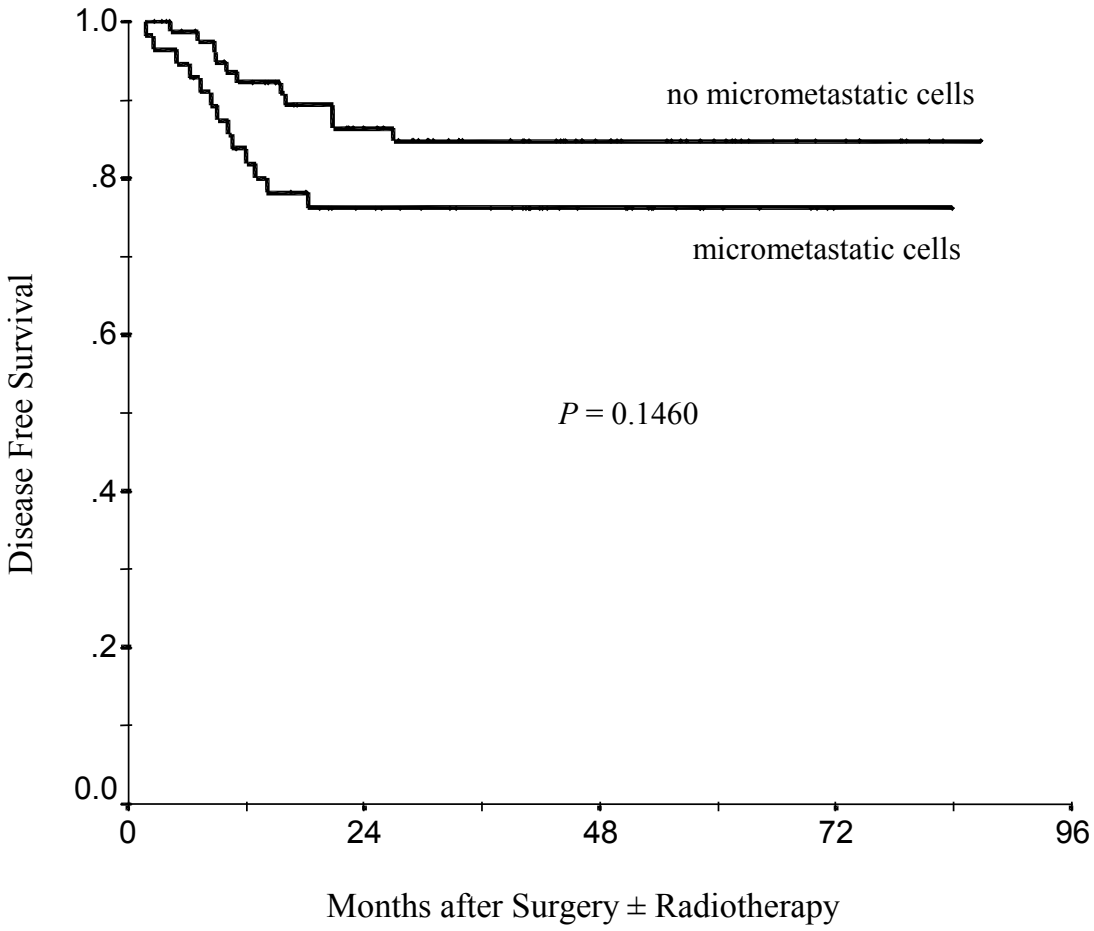
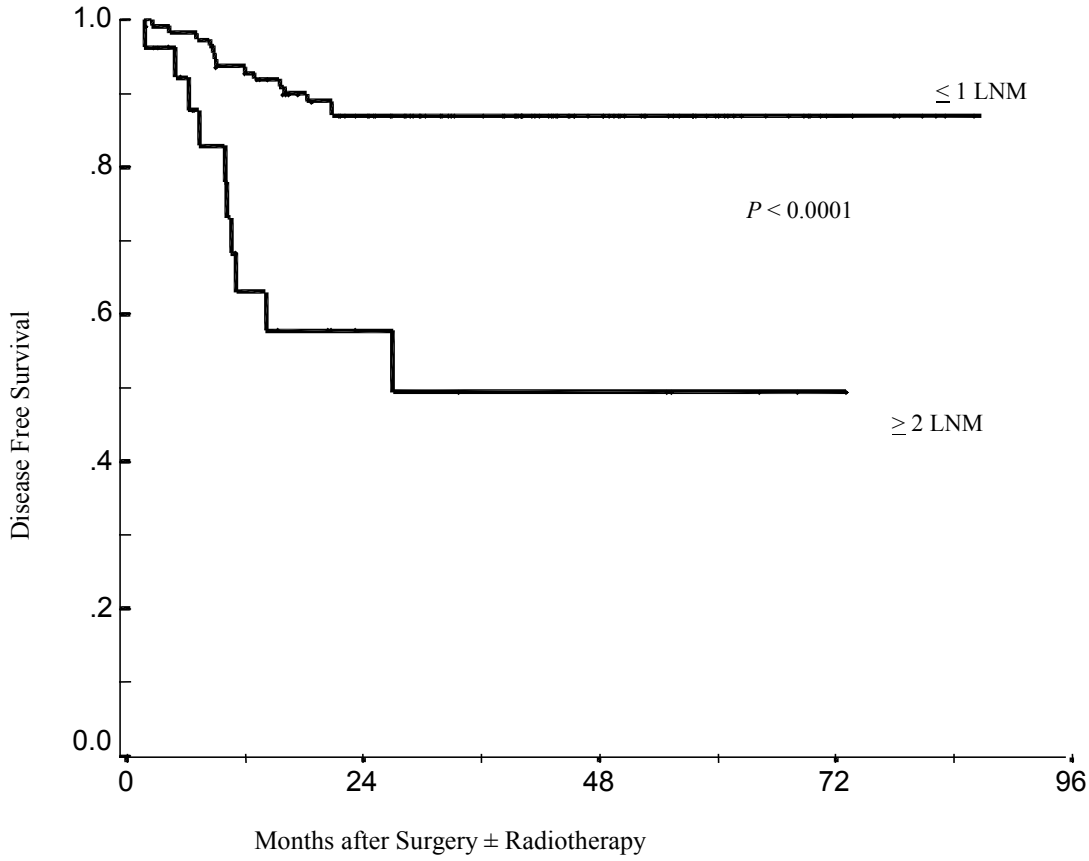


Figure 1. E48 RT-PCR of bone marrow samples of HNSCC patients. Upper panel: a serial dilution of RNA from the E48 expressing cell line UM-SCC-22A, ranging from 50, 15, 5, 1.5, to 0.5 pg, was run in parallel in each experiment as positive control. Lower panel: preparations without RNA template were used as negative RT-PCR control. In addition, a blotting control was loaded on the agarose gels consisting of dilutions of EcoRI cut plasmid DNA containing the E48 cDNA to standardize autoradiography. RT-PCR amplification of β2-microglobulin transcripts were used as control for the quality of the RNA. Four representative examples of bone marrow aspirates of HNSCC patients are depicted; samples P1 and P3 were scored as positive for micrometastatic cells in bone marrow.



No. of patients at risk				
no micro-metastatic cells	83	54	28	9
micrometastatic cells	56	35	17	1

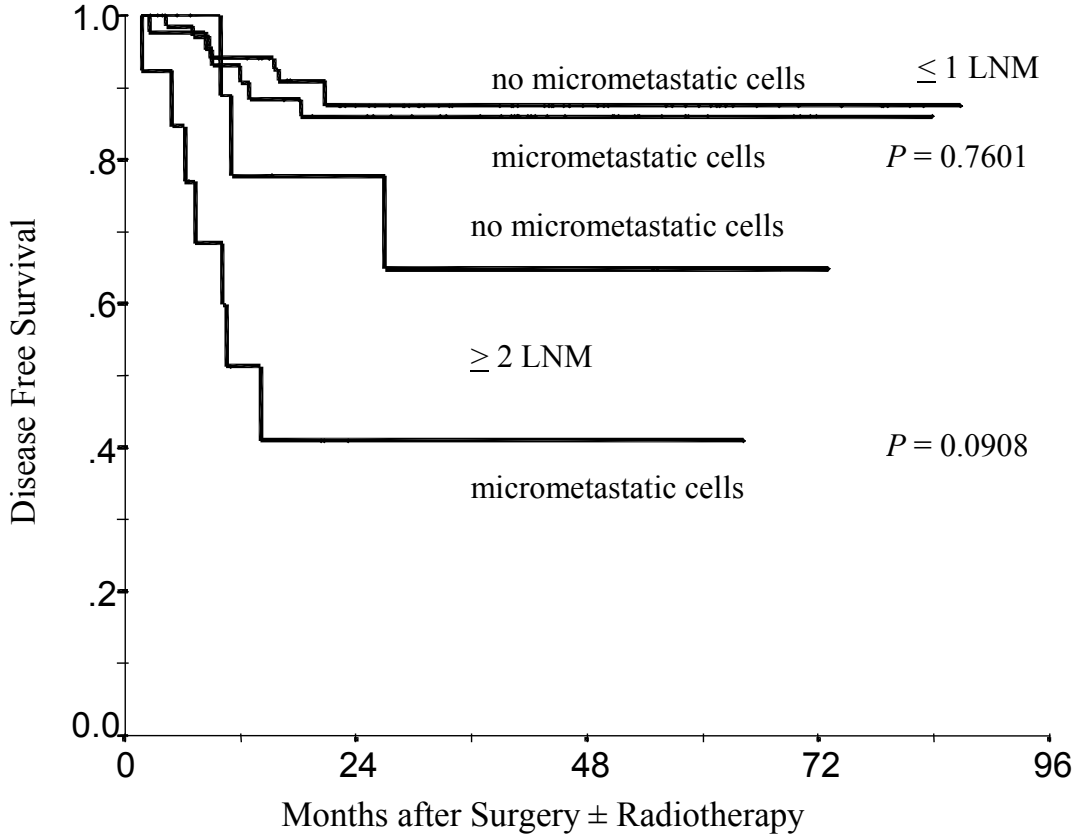
Figure 2. Kaplan-Meier life-table analysis of the disease-free survival of HNSCC patients, according to the presence or absence of micrometastatic cells in bone marrow as assessed by E48 RT-PCR at initial work-up (*P* = 0.1460).



Number of patients at risk				
≤ 1 LNM	92	77	36	7
≥ 2 LNM	47	14	9	3

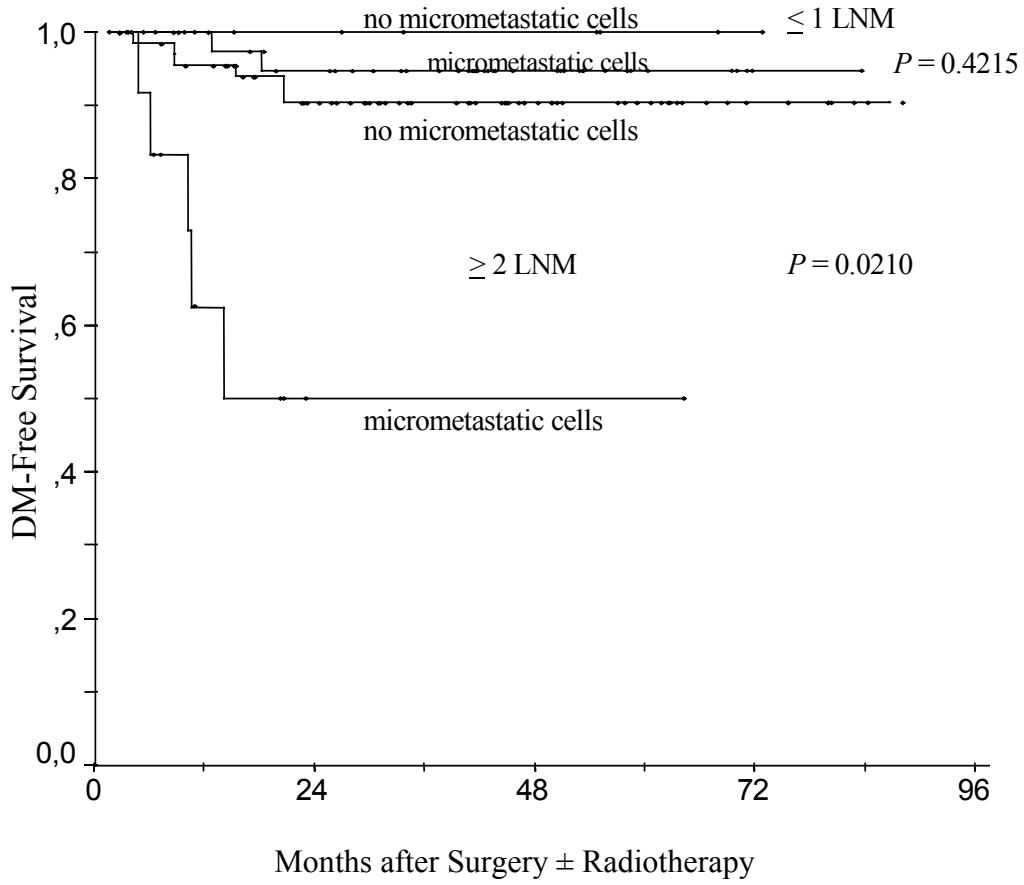
Figure 3. Kaplan-Meier life-table analysis of the disease-free survival of HNSCC patients, related to the number of lymph node metastases (LNM) as assessed by histopathological analysis of the neck dissection specimen. The disease-free survival of patients with ≤ 1 LNM versus patients with ≥ 2 LNM is significantly different (*P* < 0.0001).

Figure 4A



Number of patients at risk				
≤ 1 LNM, no micrometastatic cells	57	44	21	6
≤ 1 LNM, micrometastatic cells	35	31	15	1
≥ 2 LNM, no micrometastatic cells	26	10	7	3
≥ 2 LNM, micrometastatic cells	21	4	2	0

Figure 4B



Number of patients at risk				
≤ 1 LNM, no micro-metastatic cells	57	44	21	6
≤ 1 LNM, micro-metastatic cells	35	31	15	1
≥ 2 LNM, no micro-metastatic cells	26	10	7	3
≥ 2 LNM, micro-metastatic cells	21	4	2	0

Figure 4. Kaplan-Meier life-table analysis of the disease-free (Figure 4A) and distant metastases-free (Figure 4B) survival of HNSCC patients, related to the presence of micrometastatic cells in bone marrow as assessed by E48 RT-PCR at initial work-up and to the number of lymph node metastases (LNM) as assessed by histopathological analysis of the neck dissection specimen. Figure 4A: significant difference in disease-free survival was neither observed for patients with ≤ 1 LNM nor for patients with ≥ 2 LNM (*P* = 0.7601 and *P* = 0.0908, respectively). Figure 4B: the presence of micrometastatic cells in bone marrow of patients with ≥ 2 LNM correlated with a poor distant metastases-free survival (*P* = 0.0210). No significant difference in distant metastases-free survival was observed for patients with ≤ 1 LNM (*P* = 0.4215). Abbreviations: LNM, lymph node metastasis; DM, distant metastases.

Discussion

The E48 antigen is selectively expressed on HNSCC, and no expression has been observed on bone marrow cells (*21,24,25*). An earlier study showed no positive E48 RT-PCR results in bone marrow aspirates of noncancer controls (*18*). Therefore, the findings of E48 RNA transcripts in bone marrow seems a strong indicator for the presence of HNSCC micrometastatic cells. In this study, a positive E48 RT-PCR result in bone marrow was associated with a poor distant metastases-free survival for patients with multiple lymph node metastases at the time of primary surgery. Patients with multiple lymph nodes are known to be at risk for development of recurrent disease, and the observations of this study indicate that selection of a high-risk subgroup of patients, based on E48 RT-PCR findings, is feasible. In this way, the assay could be a useful tool to identify patients for whom adjuvant systemic treatment is recommended.

From the 83 patients with a negative E48 RT-PCR result, a total of 11 patients developed recurrent disease: 3 local recurrences, 2 regional recurrences, and 6 distant metastases. Especially the development of distant metastases should be regarded as a false-negative result of the E48 RT-PCR assay. An explanation for false-negative findings might be the heterogeneous expression of the E48 antigen. In primary HNSCC, immunohistochemical data showed homogeneous expression of the E48 antigen in 70% of primary tumors, and heterogeneous expression in an additional 19% (*26*). No data are available on E48 expression by micrometastatic HNSCC cells. False-negative findings could also be explained by sampling errors as a results of the low number of micrometastatic cells in bone marrow of HNSCC patients. This phenomenon is well known from studies with other tumor types. In breast cancer patients, it has been demonstrated that collection of four bone marrow samples instead of one increases the rate of detection of micrometastatic cells (*27*). It is very likely that the number of micrometastatic cells is even lower for HNSCC than for breast cancer patients, thus requiring multiple bone marrow sampling at different sites. It is unlikely that the sensitivity of the E48 RT-PCR assay itself is responsible for false-negative results. The level of sensitivity of the E48 RT-PCR has been assessed in seeding experiment with the HNSCC cell line UM-SCC-22A, and showed reproducible detection of a single tumor cell in a background of around 2×10^7 white blood cells (*18,20*).

A low specificity of the E48 RT-PCR would make the assay less suitable for selection of patients for adjuvant therapy. In our study, positive E48 RT-PCR results were observed in 31 of 81 patients who did not develop recurrent disease despite a sufficient median duration of follow-up. An explanation might be that these positive

E48 RT-PCR results indeed indicate the presence of micrometastatic cells, but that they represent non-proliferating or dormant metastatic tumor cells (*11*). Postoperative bone marrow sampling has been suggested for identification of persistent populations of micrometastatic cells that might be best equipped for metastasis outgrowth, but initial data do not support this (*28*).

The E48 RT-PCR assay detected micrometastatic cells in bone marrow aspirates in 40% of the patients, which is in concordance with other reports using immunocytochemistry on bone marrow aspirates in HNSCC patients (*15,16*). In these studies, an association between the presence of cytokeratin-positive cells and clinical outcome of HNSCC patients was observed. In a recent report on a smaller number of HNSCC patients by Partridge *et al.*, detection of micrometastatic cells in bone marrow with combined immunocytochemistry and E48 RT-PCR was associated with a higher risk for both local and distant relapse, as well as reduced survival (*28*). The E48 RT-PCR on bone marrow aspirates in their study was positive in just 9 of 36 patients who underwent surgery for primary HNSCC, and recurrent disease occurred in 7 of these 9 patients, of whom 4 developed distant metastases. The concordance between immunocytochemistry and E48 RT-PCR was good, and each single test predicted development of distant metastases.

We conclude that presence of micrometastatic cells in bone marrow of HNSCC patients with ≥ 2 lymph node metastases at the time of primary surgery is associated with a poor distant metastases-free survival. Nevertheless, the E48 RT-PCR assay meets limitation to select HNSCC patients for adjuvant systemic treatment because of a suboptimal specificity and sensitivity. Increasing the number and time points (postoperatively) of bone marrow aspirates might be necessary to further improve the sensitivity and specificity of the assay to be of value for selection of HNSCC patients at risk for development of recurrent disease at distant sites.

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7

Summary, Discussion and Future Perspectives

Squamous cell carcinoma is by far the most frequently occurring malignant tumor in the head and neck. More than 95% of head and neck squamous cell carcinoma (HNSCC) originate from the mucosal linings of the oral cavity, the pharynx, or the larynx. The estimated worldwide incidence of HNSCC in 2000 was more than 551,000 cases, and more than 217,000 deaths. Initial therapy of HNSCC consists of surgery and/or radiotherapy.

In the introduction **(Chapter 1)** of this thesis, it is outlined that for advanced stages III and IV HNSCC the failure rate remains high (both locoregionally and at distant sites), despite improvements in locoregional control, as the incidence of distant metastases increased. The addition of (neo)adjuvant chemotherapy to the primary treatment of HNSCC has resulted in improved quality of life, particularly in terms of organ preservation, but not in a benefit in survival of these patients. An effective systemic adjuvant treatment is therefore needed in order to improve survival rates. Among novel therapeutic approaches is the use of monoclonal antibodies (MAbs), able to recognize specific structures on tumor cells and, when loaded with toxic agents, able to kill these tumor cells. Most MAbs available nowadays, have been generated by hybridoma technology and were murine from origin. One of the major limitations of using these murine MAbs (mMAbs) in patients is immunogenicity, which can impede repeated administrations of mMAbs for multi-cycle treatment strategies. Advances in DNA technology have allowed engineering of chimeric (mouse/human) and humanized MAbs, which are expected to have reduced immunogenicity as compared to their murine counterparts.

Radioimmunotherapy (RIT) is targeted radiotherapy using tumor-selective MAbs labeled with radionuclides. In this way, systemically administered radiation can be selectively delivered to tumor sites irrespective their location in the body while sparing normal tissues. The most widely used radionuclides for RIT are ^{131}I and ^{90}Y. Based on its physical properties, rhenium-186 (^{186}Re) might be another suitable candidate. For ^{186}Re, the optimal tumor diameter for curability ranges between 7.0-12.0 mm, making it an ideal radionuclide for RIT of small tumor lesions or as adjuvant treatment (*1*). Almost all decay (91%) is by therapeutic β-emission, and a modest (9%) part is by low-energy γ-emission, which has excellent imaging properties and can be used for confirmation of tumor targeting as well as for dosimetric purposes. Knowledge of dosimetry in tumor and normal tissues is essential for an appropriate planning of RIT. In a pretherapy or scouting procedure, dosimetry can be used for individualization and increased accuracy of RIT. Since the most important dose limiting toxicity of RIT is bone marrow suppression, prediction of bone marrow toxicity with a pretherapy study might improve safety as well as efficacy.

In the last decade, RIT has proven to be particularly effective in the treatment of hematological malignancies, especially non-Hodgkin's lymphomas (NHL). The success of RIT in this field is due to several factors, among which are the intrinsic activity of the MAbs under consideration consisting of ADCC, CDC, and apoptosis induction upon binding to the target cell, together with the good accessibility for MAbs, homogeneous expression of target antigens and the high intrinsic radiosensitivity of lymphoma tumors and cells. Despite the encouraging results of RIT in patients with hematological malignancies, however, for solid tumors such results have not been achieved as yet. In the majority of studies, tumor-absorbed dose estimates have been relatively low, with few clinical responses, and bone marrow being the dose limiting organ in all studies. Strategies to enhance the therapeutic efficacy of RIT in solid tumors have been based on increasing the tumor-absorbed dose or reducing bone marrow toxicity. The latter can be achieved with the use of autologous blood or bone marrow progenitor cell transplantation.

For RIT in HNSCC patients, the v6 domain of CD44 splice variants (CD44v6) might be a promising target. From earlier clinical radioimmunoscintigraphy and biodistribution trials it was learned that the anti-CD44v6 MAbs U36 and BIWA are equally well suited for selective targeting of antigen-positive primary tumors and lymph node metastases. In view of the fact that the future role for RIT in HNSCC patients is thought to be a systemic adjuvant treatment, identification of patients at risk for development of recurrent disease is a cornerstone. Among strategies used for other solid tumors to select patients at risk is detection of micrometastatic cells in bone marrow. For HNSCC, the E48 antigen has been proposed as a suitable marker antigen for the detection of micrometastatic cells in bone marrow by RT-PCR (*2*).

The primary aim of this thesis was to investigate the feasibility of RIT with CD44v6 targeting MAbs in HNSCC patients. Besides that, the value of E48 RT-PCR on bone marrow aspirates for detection of micrometastatic cells was determined in order to identify patients at highest risk for development of recurrent HNSCC, particularly at distant sites.

In a phase I radiation dose-escalation study with ^{186}Re-cMAb U36 in HNSCC patients **(Chapter 2)**, it was demonstrated that ^{186}Re-cMAb U36 can be safely administered with the MTD established at 27 mCi/m^2 (1.0 GBq/m^2), and dose limiting bone marrow toxicity at 41 mCi/m^2 (1.5 GBq/m^2). The extent of bone marrow toxicity correlated with the bone marrow absorbed dose as determined from blood pharmacokinetics of ^{186}Re-cMAb U36. Although pharmacokinetics of ^{186}Re-cMAb U36 varied between patients, these could be predicted by ^{99m}Tc-cMAb U36, which was administered as a diagnostic imaging study prior to RIT. Moreover, scintigraphy after administration of ^{99m}Tc- and ^{186}Re-cMAb U36 showed a similar biodistribution, including comparable consistent and selective uptake at tumor sites. Due to the longer

half-life of ^{186}Re in comparison with ^{99m}Tc, improved delineation of small lesions and distant metastases was observed with ^{186}Re-cMAb U36 at later time intervals. Despite the use of a chimeric MAb, induction of human antibody responses was observed in five of thirteen patients. In patients treated at the highest radiation dose levels anti-tumor effects were observed.

The administered radioactivity dose in RIT can be fixed or to some level individualized, based on the patients' body weight or body surface area. More individualized treatment protocols make use of a pretherapy imaging study to confirm tumor targeting and/or to estimate the whole body or bone marrow absorbed dose. Although this approach might increase the accuracy and safety of RIT, it requires an extra study and is a burden for patients. For ^{186}Re-cMAb U36 RIT, a pretherapy imaging study to confirm tumor targeting is of limited value, since consistent and selective uptake at tumor sites was observed in the phase I trial described above. Moreover, the U36 antigen is expressed homogeneously in practically all HNSCC tumors, making the need for confirmation of tumor targeting superfluous. On the contrary, pharmacokinetics of cMAb U36 do vary between patients and accurate prediction of the pharmacokinetics and, consequently, the bone marrow absorbed dose of ^{186}Re-cMAb U36 RIT, can be useful. To achieve this, a pretherapy pharmacokinetic study with a limited number of blood samples is to be preferred. In **Chapter 3** a study has been described on the prediction of the total ^{186}Re-cMAb U36 pharmacokinetics by a limited number of blood samples from the therapy (^{186}Re-cMAb U36) or pretherapy (^{99m}Tc-cMAb U36) study. Moreover, it was evaluated whether myelotoxicity could be predicted by a limited number of blood samples from the therapy or pretherapy study. Population pharmacokinetic analysis by using a non-parametric expectation algorithm and Bayesian analysis of individual patient data allowed accurate prediction of the ^{186}Re-cMAb U36 clearance by limited sampling at 4 and 72 h after administration of ^{186}Re-cMAb U36. This prediction was less accurate when the 4 and 21 h blood samples from the ^{99m}Tc-cMAb U36 pretherapy study were used. Moreover, the development of bone marrow toxicity was equally well predicted by limited blood sampling in the diagnostic study with ^{99m}Tc-cMAb U36, as well as in the ^{186}Re-cMAb U36 RIT study. ^{186}Re-cMAb U36 appeared to be a better candidate for a pretherapy study as compared to ^{99m}Tc-cMAb U36, due to the longer physical half-life of ^{186}Re. As a consequence, a single type of radioimmunoconjugate can be used for both pretherapy study and RIT when individualized treatment planning is preferred.

In order to reduce bone marrow toxicity and to achieve a higher MTD of ^{186}Re-cMAb U36, transplantation of autologous blood progenitor cells was evaluated in another phase I trial with ^{186}Re-cMAb U36 **(Chapter 4)**. Bone marrow transplantation or leucapheresis are the most common procedures used in clinical RIT

to achieve this goal. Another approach is re-infusion of granulocyte colony-stimulating factor (G-CSF) stimulated unprocessed whole blood. This procedure can be performed in a routine clinical setting at low cost, since it does not require equipment and laboratory facilities for separation and cryopreservation of the blood progenitor cells. The approach of ^{186}Re-cMAb U36 RIT supported by re-infusion of G-CSF stimulated unprocessed whole blood was used successfully, and resulted in reduced bone marrow toxicity, despite a higher bone marrow absorbed dose and a doubling of the MTD to 54 mCi/m^2 (2.0 GBq/m^2). In the majority of patients as well as in all patients treated at the MTD-level stabilization of disease was observed.

A drawback of cMAb U36 is the induction of human antibody responses in a considerable number of patients, impeding multiple administrations. This problem can be circumvented by the use of humanized MAbs. The anti-CD44v6 humanized MAb BIWA 4 was therefore evaluated in a biodistribution study with ten patients who underwent surgery for primary HNSCC **(Chapter 5)**. None of the patients showed a human antibody response on administration of ^{99m}Tc-BIWA 4, and tumor uptake values were similar as previously observed for cMAb U36. In the meantime, a phase I radiation dose-escalation study with ^{186}Re-BIWA 4 in HNSCC patients has been carried out (*3*). A total of 20 patients were treated in this study, and 3 of them received multiple administrations. The MTD was established at 50 mCi/m^2 (1.85 GBq/m^2) and stabilization of disease was observed in 3 of 6 patients treated at the MTD-level, and in only 2 of 20 patients induction of human antibody responses was observed.

In order to select HNSCC patients who might benefit from adjuvant RIT, the prognostic value of the presence of micrometastatic cells in bone marrow of HNSCC patients as assessed by E48 RT-PCR on bone marrow aspirates taken was evaluated in **Chapter 6**. In 56 of 139 (40%) HNSCC patients treated with primary surgery bone marrow micrometastatic cells could be detected at initial work-up. A positive E48 RT-PCR result was not associated with poor disease-free survival. For patients with multiple lymph node metastases at the time of primary treatment, however, a significant association was found with distant metastases-free survival.

Thus far, it can be concluded that RIT with ^{186}Re-cMAb U36 is safe and tumoricidal doses can be achieved in HNSCC patients. A phase II study at the MTD level could be the next step for evaluation of the potential of RIT for treatment of HNSCC in the future. Next, the efficacy of ^{186}Re-cMAb U36 can be evaluated in a phase III clinical trial with a large group of patients. If re-infusion of G-CSF stimulated unprocessed whole blood is used in a phase II study, a higher radioactivity dose can be administered, which is an attractive option in terms of clinical responses to be expected. Although the procedure of re-infusion of G-CSF stimulated unprocessed whole blood is well tolerated by patients and can be performed at

relatively low cost, it would make the study protocol more complicated. Alternatively, evaluation of ^{186}Re-BIWA 4 in a phase II study can be regarded as next attractive step as well. ^{186}Re-BIWA 4 has demonstrated antitumor effects in a phase I dose escalation RIT study and in contrast to cMAb U36 has shown minimal development of human antibody responses, which allows repeated administration of this MAb (*3*).

Although for complete tumor eradication the radiation dose delivered to tumor lesions should be increased several times, the observation of antitumor effects in patients with bulky disease offers opportunities for further development of RIT as an adjuvant treatment for HNSCC. It has been demonstrated that the uptake of MAbs in small volume HNSCC tumors (1 mm^3) is generally about four times higher than uptake in larger tumors (50 mm^3) (*4*). Moreover, most promising results in RIT of solid tumors have been achieved in patients with small tumor lesions or minimal residual disease (*5-7*). Although not the primary aim of a phase I study, clinical responses were observed after RIT with ^{186}Re-cMAb U36, and when re-infusion of G-CSF stimulated unprocessed whole blood was used, delivery of a two times higher radiation dose to the tumor resulted in stabilization of disease in the majority of patients. Selection of HNSCC patients for systemic adjuvant therapy could be based on the extent of lymph node metastases in the neck, particularly on the number of lymph node metastases, since it has been shown that for these patients the incidence of distant metastases is increased (*8*). The risk for development of distant metastases in this subgroup of HNSCC patients is even higher when micrometastatic cells are present in bone marrow as assessed by E48 RT-PCR on bone marrow aspirates at initial work-up. Based on aforementioned data, and pending confirmation in a phase II clinical trial, we think that RIT might become a realistic option for adjuvant therapy of minimal residual HNSCC, of which the efficacy should be assessed in a large series of patients in a phase III trial.

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Samenvatting, Discussie en Toekomst

Het plaveiselcelcarcinoom is veruit het meest voorkomende maligne tumortype in het hoofd-halsgebied. Meer dan 95% van de hoofd-hals plaveiselcelcarcinomen (HHPCC) ontstaan in het slijmvlies van mondholte, keelholte en strottenhoofd. De geschatte wereldwijde incidentie van HHPCC in 2000 was meer dan 551.000 gevallen en meer dan 217.000 patiënten overleden aan de ziekte. Meestal wordt HHPCC in eerste instantie behandeld met chirurgie en/of radiotherapie.

In de introductie **(Hoofdstuk 1)** van dit proefschrift wordt beschreven dat na behandeling van gevorderde HHPCC (stadia III en IV) een hoog percentage recidief ziekte optreedt, zowel locoregionale recidieven als metastasen op afstand. Ondanks verbeteringen in de locoregionale behandeling van HHPCC met radiotherapie en chirurgie is de overleving van patiënten de afgelopen 25 jaar nauwelijks verbeterd. Dit komt met name door een toename in de incidentie van metastasen op afstand. Toevoeging van chemotherapie aan initiële radiotherapie heeft weliswaar verbetering gebracht in de kwaliteit van leven met name middels zogenaamde "organ preservation", maar tot op heden is geen verbetering in overleving bereikt.

Het gebruik van monoklonale antilichamen (MAbs) vormt een nieuwe mogelijkheid voor therapie. MAbs zijn in staat om specifieke structuren op tumorcellen te herkennen en kunnen deze vernietigen als aan het MAb een toxische stof gekoppeld is. De meeste MAbs die heden beschikbaar zijn werden met behulp van de hybridoma techniek ontwikkeld en zijn van muizen origine. Eén van de belangrijkste nadelen van het gebruik van muizen MAbs is immunogeniciteit, hetgeen herhaalde toediening in de weg kan staan. Door het gebruik van moderne DNA technologie is het mogelijk gebleken om chimere (muis/mens) en humane MAbs te ontwikkelen, welke over het algemeen minder immunogeen zijn.

Radioimmunotherapie (RIT) maakt gebruik van tumorselectieve MAbs waaraan radionucliden zijn gekoppeld. Aldus kan systemisch toegediende radioactiviteit selectief worden afgeleverd bij tumorcellen ongeacht hun lokalisatie in het lichaam. De meest gebruikte radionucliden voor RIT zijn ^{131}I en ^{90}Y. Rhenium-186 (^{186}Re) is op basis van zijn fysische eigenschappen een andere geschikte kandidaat. De optimale diameter van een tumor voor curatie met ^{186}Re RIT bedraagt 7.0-12.0 mm, hetgeen ^{186}Re tot een ideaal radionuclide maakt voor RIT van kleine tumoren (*1*). Het grootste deel van de straling die vrijkomt bij verval van ^{186}Re is β-straling (91%) met daarnaast een klein gedeelte lage energie γ-straling (9%), dat ideale eigenschappen heeft voor beeldvorming middels scintigrafie en zo gebruikt kan worden om informatie te verkrijgen over tumoropname en dosisafgifte. Dosimetrie geeft inzicht in de afgifte van straling aan tumor en normale weefsels. Als een

dergelijke diagnostische studie voorafgaand aan RIT wordt uitgevoerd spreekt men van een "pretherapie" of "scouting" studie. Pretherapie studies kunnen worden gebruikt voor zorgvuldige planning van RIT voor individuele patiënten. Omdat het beenmerg over het algemeen het orgaan is waar de meeste toxiciteit zal ontstaan (dosislimiterend orgaan), kan het voorspellen van de beenmergtoxiciteit met behulp van een pretherapie studie zowel de veiligheid als de effectiviteit verbeteren.

RIT is de laatste 10 jaar het meest succesvol gebleken bij de behandeling van lymfomen, in het bijzonder non-Hodgkin's lymfomen. Dit succes kan toegeschreven worden aan de intrinsieke activiteit van MAbs bestaande uit "antibody-dependent cellular cytotoxicity" (ADCC), "complement-dependent cytotoxicity" (CDC) en apoptose inductie na binding aan de tumorcel. Daarnaast spelen de goede toegankelijkheid van lymfomen voor MAbs, homogene expressie van de target antigenen en de intrinsieke gevoeligheid van lymfomen voor straling een belangrijke rol. RIT is helaas minder succesvol gebleken bij de behandeling van solide tumoren. In het merendeel van de studies was de stralingsdosis die aan de tumor afgegeven kon worden laag, met als gevolg dat het therapeutisch effect gering was. Strategieën om het therapeutisch effect bij solide tumoren te vergroten zijn gebaseerd op het verhogen van de stralingsdosis in de tumor en het verminderen ervan in het beenmerg. Dit laatste kan bereikt worden door gebruik te maken van beenmerg- of stamceltransplantatie.

Een veelbelovend target antigeen voor RIT van HHPCC is het v6 domein van het CD44 membraan eiwit (CD44v6). Eerdere klinische radioimmunoscintigrafie en biodistributie studies hebben laten zien dat de anti-CD44v6 MAbs U36 en BIWA in gelijke mate selectieve ophoping in primaire HHPCC tumoren en lymfkliermetastasen vertonen. Vanwege het feit dat de toekomst van RIT waarschijnlijk vooral zal liggen in het gebruik als adjuvante systemische behandeling, is het van belang dat patiënten met het hoogste risico op het ontwikkelen van recidief ziekte vooraf geselecteerd kunnen worden. Eén van de manieren om patiënten met een verhoogd risico te identificeren is door het aantonen van tumorcellen in het beenmerg. Het zogenaamde E48 antigen kan van waarde zijn voor het opsporen van deze cellen omdat dit antigeen selectief tot expressie komt in HHPCC en gedetecteerd kan worden met de zogenaamde "reverse transcriptase polymerase chain reaction" (RT-PCR) techniek (*2*).

Het primaire doel van het onderzoek zoals beschreven in dit proefschrift was het evalueren van RIT met anti-CD44v6 MAbs bij HHPCC patiënten. Daarnaast werd de waarde van het opsporen van tumorcellen in het beenmerg van HHPCC patiënten middels E48 RT-PCR voor de bepaling van het risico op recidief ziekte, met name metastasen op afstand, onderzocht.

In een fase I dosisescalatie RIT studie bij HHPCC patiënten **(hoofdstuk 2)** werd aangetoond dat ^{186}Re-cMAb U36 veilig is. De maximaal tolereerbare dosis (MTD) werd vastgesteld op 27 mCi/m^2 (1.0 GBq/m^2) en dosislimiterende beenmergtoxiciteit werd waargenomen bij 41 mCi/m^2 (1.5 GBq/m^2). De mate van beenmergtoxiciteit bleek direct gecorreleerd te zijn aan de geabsorbeerde beenmergdosis, welke bepaald werd aan de hand van de farmacokinetiek van ^{186}Re-cMAb U36 in bloed. Er trad variatie op in farmacokinetiek van ^{186}Re-cMAb U36 tussen patiënten onderling, maar de individuele kinetiek kon worden voorspeld uit pretherapie studies met ^{99m}Tc-cMAb U36. Bovendien liet scintigrafie na toediening van ^{99m}Tc- en ^{186}Re-cMAb U36 een vergelijkbare biodistributie zien, waaronder consistente en selectieve ophoping van deze conjugaten in de tumor. Tengevolge van de langere halfwaardetijd van ^{186}Re ten opzichte van ^{99m}Tc, en daardoor de mogelijkheid voor beeldvorming op latere tijdstippen, werden met ^{186}Re-cMAb U36 kleine tumoren en afstandsmetastasen duidelijker afgebeeld. Ondanks het gebruik van een chimeer antilichaam werd toch nog bij vijf van de dertien patiënten een humane-antichimeer-antilichaam reactie tegen cMAb U36 waargenomen. Bij patiënten die op het hoogste dosisniveau werden behandeld werd de tumorgroei tot stilstand gebracht.

De toegediende radioactiviteitdosis voor RIT kan een vaste dosis zijn, of een dosis die gebaseerd is op het gewicht of de lichaamsoppervlakte van de patiënt. Een meer geïndividualiseerde behandeling kan uitgevoerd worden met behulp van een pretherapie studie, waarbij de behandeldosis wordt vastgesteld op basis van middels beeldvorming waargenomen tumorophoping van het conjugaat en de stralingsafgifte op het totale lichaam of op het beenmerg. Ofschoon met zo'n pretherapie studie de RIT dosis nauwkeuriger bepaald kan worden, betekent het een extra onderzoek en dus een grotere belasting voor de patiënt. Een dergelijke pretherapie studie is in het geval van ^{186}Re-cMAb U36 RIT niet echt noodzakelijk, omdat tot nu toe bij alle patiënten een consistente en selectieve tumor opname werd gezien. De consistente tumor opname heeft onder andere te maken met het feit dat het U36 antigeen in vrijwel alle HHPCC tumoren homogeen tot expressie wordt gebracht. De farmacokinetiek van ^{186}Re-cMAb U36 varieert daarentegen wel tussen patiënten onderling met als gevolg verschil in beenmergdosis. Daarom lijkt een pretherapie studie waarbij uitsluitend naar de farmacokinetiek wordt gekeken eventueel wel nuttig. Idealiter zou de farmacokinetiek van ^{186}Re-cMAb U36 aan de hand van een beperkt aantal bloedmonsters in een pretherapie studie voorspeld moeten kunnen worden, waardoor de belasting voor de patiënt minimaal is. In **hoofdstuk 3** wordt een studie beschreven, waarin werd getracht de totale ^{186}Re-cMAb U36 farmacokinetiek te voorspellen aan de hand van een beperkt aantal bloedmonsters voor zowel de therapie (^{186}Re-cMAb U36) als pretherapie studie (^{99m}Tc-cMAb U36). Tevens werd nagegaan in hoeverre de beenmergtoxiciteit met een beperkt aantal bloedmonsters uit de therapie of

pretherapie studie betrouwbaar te voorspellen is. De ^{186}Re-cMAb U36 farmacokinetiek kon nauwkeurig voorspeld worden op basis van slechts twee bloedmonsters genomen op 4 en 72 uur na toediening van ^{186}Re-cMAb U36, door het gebruik van een non-parametrisch verwachtingsalgoritme en analyse volgens Bayes. Deze voorspelling was minder nauwkeurig als hiervoor de 4 en 21 uur bloedmonsters van de ^{99m}Tc-cMAb U36 pretherapie studie genomen werden. De mate van beenmergtoxiciteit kon bovendien evengoed worden voorspeld door ^{99m}Tc-cMAb U36 en ^{186}Re-cMAb U36. ^{186}Re-cMAb U36 bleek echter een betere kandidaat voor het voorspellen van de farmacokinetiek vanwege de langere halfwaardetijd van ^{186}Re. Dit laatste betekent dat, indien individuele therapie voorspelling wenselijk wordt geacht, voor pretherapie studie en RIT een zelfde conjugaat gebruikt kan worden.

In een andere fase I studie met ^{186}Re-cMAb U36 werd het gebruik van re-infusie van "granulocyte colony-stimulating factor" (G-CSF) gestimuleerd autoloog volbloed geëvalueerd om zo de beenmergtoxiciteit te verminderen en de MTD te kunnen verhogen **(Hoofdstuk 4)**. Voor dit doel wordt in het algemeen beenmergtransplantatie of leucaferese aangewend. Re-infusie van gestimuleerd autoloog volbloed is echter een interessant alternatief, omdat het in vrijwel ieder ziekenhuis toegepast kan worden en relatief goedkoop is. Omdat bij deze techniek geen beenmergstamcellen gezuiverd en opgeslagen worden, zijn geen dure laboratoriumfaciliteiten nodig. Ondersteuning van ^{186}Re-cMAb U36 RIT door re-infusie van G-CSF gestimuleerd autoloog volbloed bleek succesvol en de beenmergtoxiciteit kon aanzienlijk worden verminderd, waardoor een verdubbeling van de MTD tot 54 mCi/m^2 (2.0 GBq/m^2) kon worden bereikt. Bij de meerderheid van de patiënten, en bij alle patiënten die op het MTD niveau werden behandeld, werd stabilisatie van de ziekte waargenomen.

Een nadeel van het gebruik van cMAb U36 is het optreden van humane-antichimeer-antilichaam reacties in een aanzienlijk gedeelte van de patiënten, hetgeen herhaalde toediening van cMAb U36 in de weg staat. Deze beperking wordt over het algemeen minder waargenomen als gehumaniseerde MAbs gebruikt worden. BIWA 4 is een gehumaniseerd anti-CD44v6 MAb dat werd geëvalueerd in een biodistributie studie met tien patiënten die werden geopereerd vanwege een primair HHPCC **(Hoofdstuk 5)**. Er werden geen humane antilichaam reacties waargenomen na toediening van ^{99m}Tc-BIWA 4 en de opname van het MAb in tumor en normale weefsels bleek vergelijkbaar met die van cMAb U36. Inmiddels is ook een fase I RIT studie met ^{186}Re-BIWA 4 in 20 HHPCC patiënten uitgevoerd (*3*), waarbij drie patiënten het BIWA 4 meer keren kregen toegediend. Humane antilichaam reacties traden slechts bij twee patiënten op. De MTD bleek 50 mCi/m^2 (1.85 GBq/m^2) te zijn en stabilisatie van de ziekte werd gezien in drie van de zes patiënten die werden behandeld op het niveau van de MTD.

De waarde van het opsporen van tumorcellen in het beenmerg van HHPCC patiënten middels E48 RT-PCR voor de bepaling van het risico op recidief ziekte, met name metastasen op afstand, is beschreven in **hoofdstuk 6**. In 56 van de 139 (40%) patiënten die werden geopereerd vanwege een primair HHPCC konden preoperatief tumorcellen in het beenmerg worden gedetecteerd. Een positieve E48 RT-PCR test bleek niet gecorreleerd met ziekte-vrije overleving. Er werd wel een significant verband gevonden tussen een positieve E48 RT-PCR test en het optreden van metastasen op afstand voor patiënten die bij operatie twee of meer tumorpositieve lymfklieren in de hals hadden.

Uit de studies beschreven in dit proefschrift kan worden geconcludeerd dat RIT met ^{186}Re-cMAb U36 veilig is en dat bovendien klinische responsen werden waargenomen bij HHPCC patiënten. De volgende stap zou een fase II studie kunnen zijn waarin de effectiviteit wordt vastgesteld in een groter aantal patiënten, behandeld op het niveau van de MTD. Vervolgens kan de effectiviteit van ^{186}Re-cMAb U36 als adjuvante therapie in een grote groep HHPCC patiënten (fase III) worden onderzocht. Door toepassing van re-infusie van G-CSF gestimuleerd autoloog volbloed in een fase II studie, kan een hogere MTD worden gebruikt en mag een beter klinisch resultaat worden verwacht. Ofschoon re-infusie van G-CSF gestimuleerd autoloog volbloed relatief eenvoudig is uit te voeren en de belasting voor patiënten aanvaardbaar is, zal het RIT studieschema uitgebreider worden. Als alternatieve fase II studie kan worden gedacht aan BIWA 4, dat als ^{186}Re-BIWA 4 in een fase I studie stabilisatie van de ziekte tot gevolg had bij een aantal patiënten. Bovendien is BIWA 4 nauwelijks immunogeen gebleken in tegenstelling tot cMAb U36, hetgeen herhaalde toedieningen mogelijk maakt (*3*).

Voor complete en langdurige responsen dient de stralingsafgifte op de tumor bij RIT nog enkele malen hoger te worden dan nu het geval is. Het feit dat antitumor effecten werden waargenomen in de beschreven studies is gunstig, omdat het hier over het algemeen patiënten betrof met grote tumoren of metastasen. Het is bekend dat opname van MAbs in kleine HHPCC tumoren (± 1 mm^3) gemiddeld vier keer hoger is dan opname in grote tumoren (± 50 mm^3) (*4*). Bovendien hebben RIT studies bij patiënten met solide tumoren de beste resultaten laten zien wanneer het patiënten betrof met kleine tumoren of minimale residuele ziekte (*5-7*). In de studie met re-infusie van G-CSF gestimuleerd autoloog volbloed (hoofdstuk 4) werd aangetoond dat een verdubbeling van de stralingsafgifte aan de tumor resulteerde in stabilisatie van de ziekte in het merendeel van de patiënten. De selectie van patiënten voor adjuvante RIT kan gebaseerd zijn op klinische criteria zoals de aanwezigheid van multipele tumorpositieve lymfklieren in de hals, omdat deze patiënten een grote kans op recidief ziekte, in het bijzonder metastasen op afstand, hebben (*8*). Ofschoon de E48 RT-PCR test op beenmerg een subgroep van HHPCC patiënten kan aanwijzen

met een verhoogd risico op het ontwikkelen van metastasen op afstand, lijkt verder onderzoek in de toekomst noodzakelijk om tot een test te komen die gebruikt kan worden voor de selectie van HHPCC patiënten voor wie adjuvante therapie in aanvulling op operatie en/of bestraling wenselijk is. Gebaseerd op de bovenstaande gegevens en in afwachting van de resultaten van een fase II studie, lijkt ons verdere evaluatie van RIT als adjuvante systemische therapie voor HHPCC gerechtvaardigd.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 17 februari 1971 te Bergen (NH). Na het behalen van het eindexamen aan het Murmellius Gymnasium te Alkmaar werd in 1989 begonnen met de studie geneeskunde aan de Universiteit Utrecht. Tijdens de studie deed hij gedurende een jaar wetenschappelijk onderzoek bij de vakgroep Functionele Anatomie van de faculteit geneeskunde van de Universiteit Utrecht (begeleiders dr. R.L.A.W. Bleys en dr. G.J. Groen) en het Department of Anatomy and Developmental Biology van de Royal Free Hospital School of Medicine te London (begeleider dr. T. Cowen). Het artsexamen werd behaald in 1997. In november van dat jaar startte hij zijn promotieonderzoek op de sectie Tumorbiologie van de afdeling Keel-, Neus- en Oorheelkunde van het VU medisch centrum Amsterdam, onder supervisie van prof.dr. G.A.M.S. van Dongen en prof.dr. G.B. Snow. Voor dit onderzoek werd hem in 1999 de Woldring prijs van de Nederlandse Vereniging voor Nucleaire Geneeskunde uitgereikt. In mei 2000 startte hij met de opleiding tot keel-, neus- oorarts in het VU medisch centrum Amsterdam (opleiders prof.dr. G.B. Snow en prof.dr. C.R. Leemans). Een deel van deze opleiding werd respectievelijk in het Diakonessenhuis Utrecht (opleider dr. J.J. Quak) en het Ziekenhuis Hilversum (opleider dr. M.J. Middelweerd) gevolgd.